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(71) Applicant *(for all designated States except US)*: SEQ, LTD. [US/US]; 17 Princess Road, Lawrenceville, NJ 08648 (US).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: MACKLIN, John, J. [US/US]; 104 North Monroe Avenue, Wenonah, NJ 08090 (US). TRAUTMAN, Jay, K. [US/US]; 28 East Welling Avenue, Pennington, NJ 08534 (US). HARRIS, Timothy, D. [US/US]; 1387 Church Road, Toms River, NJ 08755 (US).

(74) Agents: MORRIS, Francis, E. et al.; Pennie & Edmonds LLP, 1155 Avenue of the Americas, New York, NY 10036 (US).

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(54) Title: METHOD TO MAKE FLUORESCENT NUCLEOTIDE PHOTOPRODUCTS FOR DNA SEQUENCING AND ANALYSIS

(57) Abstract

A method is described for making fluorescent photoproducts of nucleotides using certain solvents in degassed solutions under ultraviolet illumination. These photoproducts can be highly fluorescent (quantum yield QY on order of 50 %) in room temperature aqueous solutions and over a wide pH range, and have low bleaching probability on order of 10^{-4}, with improvement anticipated with the use of triplet quenchers. These modified nucleotides, when bound and immobilized on an alumina surface, can be detected at the single molecule level. Their value lies in the significant enhancement in the detectability of nucleotides by fluorescence, and may offer an attractive set of fluorphores for application to base–at–a–time DNA sequencing.

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METHOD TO MAKE FLUORESCENT NUCLEOTIDE PHOTOPRODUCTS FOR DNA SEQUENCING AND ANALYSIS

Field of the Invention

The present invention relates to the formation of fluorescent products of native nucleotides as a result of a photochemical reaction, and the use of these photoproducts for enhanced detection of nucleotides by fluorescence. It additionally relates to the binding of these nucleotides to surfaces, where immobilization facilitates their detection at exceedingly low concentrations.

Background of the Invention

The development of a high throughput DNA sequencer capable of long read lengths that can generate DNA sequence information at low cost would have a significant impact on the drug discovery process and the understanding of the genetic basis of disease. One such method to sequence DNA takes advantage of the sequential cleavage of bases from an oligonucleotide by a processive exonuclease, where the sequence is determined by the detection and discrimination of the ordered cleaved bases. Such a base-at-a-time sequencing device, operated at high throughput (10-100 bases/sec) on long DNA strands (20-40 kilobase lengths), depends fundamentally on the reliable fluorescence detection of nucleotides at the single molecule level.

Reliable detection of nucleotides requires that the emitting molecule appear brighter than the background emission of the matrix in which it is located. After methods are employed to minimize the background fluorescence, the molecule must emit a sufficient number of photons to be distinguishable from detector noise. Based on practical experience and calculations of detection limits, we find that in the ultraviolet wavelength range where the nucleotides absorb and emit, the nucleotide fluorescence quantum yield (the fractional number of fluorescent photons emitted per absorbed photon) would preferably exceed 50%, and the

molecule must emit enough photons so that at least 100 photons are detected per nucleotide, before undergoing any irreversible decomposition which destroys its emitting properties, for example by photobleaching. This would require, for a detection geometry with 2.5% collection/detection efficiency, 4000 photons emitted per nucleotide before bleaching. Since bleaching is probabilistic, in order to insure that, say, 90% of the molecules survive the bleaching process to yield at least 4000 photons, the actual bleaching probability should be less than about 2×10^{-5}. Any analysis of single molecule detection which fails to address this photostability issue will arrive at fundamentally flawed conclusions.

The four common nucleotides (A, C, G, T) are virtually non-fluorescent under physiological conditions, such as those where a single DNA strand is to be sequentially cleaved by an exonuclease, with quantum yields in the range of 0.01%. Only at acid pH will G and to a much smaller degree A show any fluorescence, with a typical quantum yield for G of 2% in a room temperature, aqueous solution at pH 1.68. This should be referred to as an average quantum yield, since the fluorescence decay is not a single-exponential, indicating a distribution of quantum yields. The quantum yields of the four nucleotides preclude single molecule detection in room temperature solutions.

It is known that in cold, rigid matrices nucleotide fluorescence can be substantially increased, with quantum yields of about 15% for G and T, 5% for C, and < 1% for A. We have shown that G in a room temperature, low pH glass formed by a spin-coating method, has an apparent quantum yield of about 15%, which increases to about 50% at 77 °K. Other nucleotides also show increased yield, for example T is 1% in a room temperature glass, which increases to about 8% at 77 °K. So the enhancement of quantum yield of nucleotides is mainly due to the rigidity of the matrix, but an additional improvement is obtained at very cold temperatures. It is possible, although very challenging, to demonstrate

single nucleotide detection based on the quantum yields of about 10%. Therefore it is possible that a single G and/or T might be detected in a rigid matrix, particularly at low temperature.

However, a significant limitation arises for molecules in rigid matrices, which severely limits the number of photons emitted. Nucleotides excited to their emitting state have a small probability of decaying to a metastable triplet state, which at cold temperatures may live for a fraction of a second before decaying back to the ground state, from where the molecule can then be excited back up to the emitting state. During the time the molecule spends in the metastable triplet state, it can react with the surrounding matrix, and decompose as a result, or it can absorb some of the incident uv light, and get promoted to a new state which can ionize or dissociate, again leading to decompostion and irreversible bleaching. The rate of these decomposition processes in the former case is linear in uv intensity while in the latter case it depends on the square of the intensity. The nonlinear bleaching can be mitigated by lowering the intensity, and we have demonstrated a method to perform simultaneous detection of many individual molecules in an imaging detection arrangement, which allows substantial reduction in excitation intensity without loss in sequencing speed (*i.e.*, the number of detected nucleotides per second). For the bleaching that is linear in intensity, no change in uv intensity will mitigate the bleaching, because the fluorescence is linear in intensity also.

It is well known that the triplet state can be quenched by other molecules through an energy transfer process, and this reduces the triplet lifetime and therefore the chance a decomposition occurs. For example, oxygen is a triplet quencher, but is potentially undesirable because when oxygen accepts energy from a nucleotide triplet state, it may form a radical which subsequently attacks and decomposes the nucleotide. Other quenchers are known, for example, hexadien-1-ol, which appear to not form radicals. A key

physical condition for effective triplet quenching is the proximity and orientation of the quencher molecule with respect to the triplet-excited nucleotide. At low temperatures and in highly viscous or rigid matrices where molecules cannot easily move or reorient, the triplet quenching process is shut down, and ineffective as a method to shorten the triplet lifetime. The conditions of rigidity and low temperature that enhance the nucleotide fluorescence make triplet quenching very difficult. Our measurements on G and T at low temperature indicate that less than a few photons can be detected per molecule before irreversible bleaching occurs.

Finally, if a cold, rigid unreactive matrix could be found where no bleaching resulted when the nucleotide was excited into the triplet state, the rate at which the molecule could be made to emit photons will be very low, although in principle the total number of photons emitted might be large. For any molecule there is a saturation intensity, below which the rate of emitted photons increases with excitation intensity, and above which the emitted rate of photons become constant. This saturation is again due to the fact that a molecule in the long lived triplet state does not emit photons. It can be shown that the emission rate is limited to approximately $QY/(t_2 + P_{isc}t_{triplet})$, where P_{isc} is the probability to populate the triplet and typically 0.1 for the nucleotides, $t_{triplet}$ is the lifetime of the triplet state and typically 0.3 sec at 77 °K, t_2 is the emitting state lifetime, typically 10^{-8} sec, and QY is the fluorescence quantum yield of the nucleotide, say 15%. The emission rate is then about 5 photons/sec. With a collection efficiency of 2% (typical), it would take 1000 sec to record a total of 100 photons per nucleotide. There would be little competitive advantage to single-molecule sequencing under this set of circumstances.

The considerations above motivate attempts to modify the nucleotides to enhance the fluorescence properties, principally increased quantum yield, and resistance to bleaching or decomposition, *i.e.*, increased photostability.

An additional requirement for a sequencer is that the modified nucleotides can be discriminated, by either a spectroscopic characteristic such as absorption or emission maxima, fluorescence lifetime, or a physical property which in the presence of a driving force, leads to a characteristic response, such as electrophoretic mobility. Modification of the nucleotides prior to the sequential enzymatic cleavage step is undesirable as it is time-consuming, has not been shown to work with a processive enzyme, and can introduce errors in the sequence under study. An alternative involves modification of the nucleotide following the cleavage step, either while the nucleotides are freely diffusing toward a binding surface, or after they are immobilized on a binding surface.

Summary of the Invention

A solution containing a native nucleotide and a non-fluorescent reagent is illuminated with electromagnetic radiation which results in a photochemical reaction that yields a fluorescent analog of the native nucleotide. This fluorescent photoproduct is then useful for the quantitative analysis of the native nucleotide by fluorescence detection methods.

A photochemical reaction is considered to consist of the following microscopic processes: (1) absorption of light resulting in the formation of an electronically excited state, either of the nucleotide or of the reagent, (2) primary photochemical reaction involving the excited nucleotide and reagent, or excited reagent and nucleotide, that form intermediate molecules, followed by (3) thermally-driven reactions of the intermediates to stable products. When the photochemistry arises from an excited state of the nucleotide, this method can be a highly specific means to essentially fluorescently-label native nucleotides. For the purposes of DNA sequencing by single-molecule fluorescence detection, this reaction can be run on nucleotides bound to

and immobilized on a surface, or while nucleotides diffuse to such a surface.

The nucleic-acid components preferably are the 5′-monophosphate nucleotides (dAMP, dCMP, dGMP and TMP). Ideally these nucleotides are sequentially cleaved one base at a time from an oligonucleotide by a processive enzyme, and immobilized on a surface in the order in which they were cleaved, so that spatially-resolved fluorescence detection and identification of single nucleotides can then be used to determine the original sequence of cleaved nucleotides, and thus the DNA sequence of the original oligonucleotide.

The reagents to be used in the photochemistry are organic molecules such as alcohols, amines, or other hydrogen-atom donors, that are ideally non-fluorescent, so as to not obscure or limit the detection of fluorescence from the nucleotide photoproduct. Since photochemistry from reactants to products often takes place via radical intermediates, formed initially by hydrogen- or electron-abstraction from a donor reagent molecule, reagents for photochemistry may be chosen from a list of hydrogen-atom donating molecules that includes alcohol-, sugar-, amine-, hydrocarbon-containing compounds, and this list includes nucleic acid components as well. Since the photochemistry may proceed where an active reagent absorbs the uv light, followed by the abstraction of a hydrogen atom from the nucleotide, reagents that are good hydrogen-atom acceptors, such as aromatic ketones like benzophenone, may also be useful. The principal issue in the choice of the reagent is the specificity of the photochemical reaction to produce a fluorescent product of a nucleotide, while additionally that product should not undergo photochemical reactions itself.

As a general rule, photochemical reactions usually proceed from the triplet state of a molecule. For chemical reactions, a long-lived triplet state is desirable to increase the time for encounters with reagent molecules. Since oxygen is a known triplet quencher, nitrogen-purged or otherwise anaerobic solutions, as well as methods to enhance

the intersystem crossing rate, should increase the photochemical quantum yield.

The conversion of a native nucleotide to a fluorescent product is accomplished using electromagnetic radiation to selectively deposit energy in the reactant molecules, and initiate the subsequent dark (or thermal) chemical reactions. Photochemical reactions, and more generally reactions proceeding from a molecular excited state, can access states which are energetically unfavorable in thermal reactions at any realistic temperature, while minimizing undesirable side reactions.

The detection of single nucleotides that have been photochemically modified can be facilitated by immobilization on a surface. A convenient and general binding motif for the nucleotides is the electrostatic attraction between the monophosphate and the surface ions of a metal-oxide film. This binding arrangement can immobilize nucleotides for hours to days, and can be shown to have little deleterious effect on the fluorescence properties of the bound nucleotides. The surface-bound molecules can be advantageously excited using a total-internal-reflectance geometry, which minimizes excitation and hence background fluorescence of the solvent. The nucleotide fluorescence can be detected in either a full-field imaging, or by using confocal detection.

Brief Description of the Drawings

These and other objects, features and advantages of the invention will be more readily apparent from the following detailed descriptions of the invention in which:

Fig. 1 is a flowchart depicting a preferred embodiment of the invention;

Figs. 2(a) and 2(b) depict experimental setups used in the practice of the invention;

Figs. 3(a) through 3(f) are plots of absorbance and fluorescence intensity versus wavelength;

Figs. 4(a) and 4(b) are plots of absorbance and fluorescence intensity versus wavelength;

Figs. 5(a) and 5(b) are plots of absorbance and fluorescence intensity versus wavelength;

Figs. 6(a) and 6(b) are plots of fluorescence intensity versus wavelength;

Fig. 7 is a plot of photocounts versus time;

Fig. 8 is a plot of fluorescence intensity versus pH;

Fig. 9 is diagrams of several molecules;

Figs. 10(a) and 10(b) are plots of fluorescence intensity versus time;

Fig. 11 is a plot of photocounts versus time; and

Figs. 12(a) and 12(b) are CCD images and linecuts of single nucleotides on a surface.

Detailed Description of the Invention

Fig. 1 is a flowchart depicting a preferred embodiment of the invention. As indicated in box 10, a nucleotide is first contacted with an essentially non-fluorescent reagent. As indicated in box 20, the nucleotide and reagent are then illuminated with electromagnetic radiation, typically in the ultraviolet part of the spectrum, to form a conversion product. As indicated in box 30, the conversion product is then illuminated with electromagnetic radiation to stimulate fluorescence. And, as indicated in box 40, the fluorescence is detected by a suitable detection system, typically a CCD camera, a spectrometer-coupled CCD or a microchannel plate detector.

Numerous variations may be made in practicing the invention. For example, it may be carried out in whole or in part on a thin film solution on a substrate. The nucleotide may be freely moving or bound to a surface such as a substrate. And the illumination steps indicated in boxes 20 and 30 may be performed as a single continuous step. Advantageously, to permit detection of fluorescence from the conversion product using presently available equipment, the conversion product should have a quantum yield of at least 10% and it should emit at least 1000 photons before photo bleaching. Preferably, the quantum yield and the photon

emission should be much higher with the quantum yield about 50% and the number of photons emitted before photo bleaching in the range of 10,000.

Experimental:

Photochemistry of nucleotides, and analysis of the fluorescent products, was performed using the experimental arrangements shown in Figs. 2(a) and 2(b). In the first arrangement shown in Fig. 2(a), large quantities of photoproduct are made by uv-illuminating a cuvet containing native nucleotides in an aqueous alcohol solution. The apparatus comprises an ultraviolet laser 70, a lens system 72, a quartz cuvet 74, a gas inlet 76 and a vent 78. This arrangement allows us to isolate the conditions that optimize the photochemistry process from those that optimize the fluorescence properties of the nucleotide photoproduct (the quantum yield, photostability, spectral characteristics, etc), since the cuvet containing the solution can be conveniently removed and analyzed for changes in absorbance and fluorescence using a spectrophotomer and a luminescence spectrometer (not shown). The change in absorbance versus the absorbed uv energy then gives the photochemical quantum yield (the probability of converting a nucleotide molecule into a photoproduct molecule per absorbed uv photon). These bulk solutions were used as stock solutions from which aliquots of photoproduct could be taken and tested versus pH, etc.

In a second arrangement shown in Fig. 2(b), photochemical reactions were performed in a thin film solution containing nucleotides and reactive solvent covering a quartz substrate, all located on a microscope stage. This apparatus comprises one or more sources of a beam of ultraviolet radiation (not shown), a lens system 102, a quartz substrate 104 bearing a thin film solution 106, a CCD camera 108, a spectrometer 110 coupled to a second CCD camera 112, a uv-enhanced microchannel plate detector 114, a beam-

splitter 116, removable mirrors 118, 120, and filters 122, 124.

This arrangement was also used to measure the fluorescent properties of nucleotides bound to a surface at a liquid/solid interface. Either epi-illumination or evanescent-wave excitation was used. For epi-illumination all of the solution can be excited, while for evanescent-excitation, only molecules on or within about 0.03-microns of the quartz surface are excited. Photoproduct fluorescence was monitored by either the CCD camera, onto which the sample fluorescence was imaged, by the spectrometer -coupled CCD for spectrally-resolved fluorescence measurements, and by the microchannel plate detector, for time-resolved measurements.

Nucleotide monophosphates were purchased from Sigma or Aldrich (>99% purity) and used as received. Dilute solutions of less then 0.1 mM concentrations typically were used. Reagents for photochemistry experiments included glycerol, 2-propanol, and ethylene glycol. The 2-propanol was sufficiently free from fluorescent impurities to be used as received, while the other solvents contained fluorescent impurities. These solvents were diluted in ultrapure water to 60 % by volume, treated with activated charcoal, filtered through 0.22-micron filters, and declared clean when, using 260 nm excitation, the impurity fluorescence at 400 nm was less than 1/10 the Raman emission at 268 nm.

The principal source of ultraviolet light was a Ti:sapphire laser, frequency-tripled into the uv, that produced tunable wavelengths from 260 nm up to 295 nm with 100-200 mWatts of power. Other sources used included an Ar-ion laser that produced 3 mW at 275 nm and 10 mW at 300 nm.

Fluorescence lifetime of samples after photoconversion was determined by time correlated photon counting, using the frequency-tripled, mode-locked Ti:sapphire laser with a pulse repetition rate of 82 MHz and a pulse width of 100 fsec and the uv-enhanced microchannel plate detector. Fluorescence quantum yield was determined by absorbance and fluorescence measurements of the photoproduct referenced to a known

standard, 2aminopurine (2AP), which has a quantum yield of 68% in water with pH 7 phosphate buffer, and 95% in unbuffered water.

In most of the photochemistry experiments, we purged the solution with nitrogen (for example, by bubbling) to displace dissolved gases, particularly oxygen. Nitrogen purging was found to significantly increase the photochemical quantum yield and decrease the photobleaching of the photoproduct over that found for air-saturated solutions. While the exact photochemical pathway that takes the nucleotide to the photoproduct is not known, it is believed to involve the nucleotide triplet state, so as a general guide for optimizing photoconversion, methods to increase the triplet yield or increase the triplet lifetime tend to enhance the photochemical yield.

Results

Figs. 3(a)-3(f) show the absorption spectra before and after uv illumination, and the fluorescence emission and excitation spectra taken after uv illumination, for various dGMP concentrations in an unbuffered solution of 30% glycerol in water. Illumination conditions were 50 mW of 275 nm light. The absorption measurements show the characteristic decrease in the parent (dGMP) absorption with the appearance of a new feature, with maxima at 305 nm and 220 nm. Clean isosbestic points can be seen. The fluorescence emission, essentially zero before illumination, shows bright fluorescence after illumination, with an emission maximum of 365 nm, and excitation maxima at 220 nm, 248 nm, and 303 nm. The excitation spectrum agrees with the photoproduct absorption features. The main difference between the three concentrations is the relative photoconversion yields.

Consider first the absorption measurements for the 15 uM dGMP solution in Fig. 3(e). Based on the change in absorbance at 260 nm, reduction in the parent dGMP of 45% means about 7 uM of G was photoconverted (due to the fact that the photoproduct has some absorption at 260 nm, the

fraction of dGMP converted is slightly higher, 55%, or about 8 uM). The increase in absorbance at 303 nm, 0.05, for a photoproduct concentration of 8 uM, yields a molar absorbtivity of about 6500 $M^{-1}cm^{-1}$ at 303 nm. For comparison, 2-aminopurine (2AP) has an extinction coefficient 7150 $M^{-1}cm^{-1}$ at 303 nm. Considering the uv energy absorbed (7.4 Joules), and the number of molecules converted (8 uM), the photochemical quantum yield was 0.12%.

As shown in Figs. 3(a), 3(c) and 3(e), the fraction of dGMP lost and photoproduct formed appears to decrease with increasing dGMP concentration. For 30 uM dGMP (Fig. 3(c)), 33% of the parent compound was converted (about 10 uM) after 20 minutes of illumination , and the absorbance at 303 nm grew to 0.10, while for 70 uM dGMP (Fig. 3(a)), 18% of the parent compound was converted (about 12.4 uM) after 24 minutes of illumination, and the absorbance at 303 nm grew to 0.15. From this data, the change in absorbencies are less than linear with concentration. Additionally, at millimolar concentrations of nucleotides (not shown), the photochemical yield is reduced by greater than 10 fold. These results indicate that the photochemistry does not involve a bi-molecular process between two nucleotides, hence our results may be scaled to very low concentrations, **i.e.**, single nucleotides.

The emission and excitation spectra of the photoproduct formed in the glycerol/water solvent are shown for the various starting concentrations of dGMP in Figs. 3(b), 3(d), and 3(f). Note the excitation spectra at the higher concentrations is uncorrected for the optical thickness of the solution in the 230-280 nm range, which artificially shifts the 303-nm absorption out to 307 nm. The photoproducts for the three concentrations are spectrally indistinguishable, and are quite similar to that of 2-aminopurine (2AP, a highly fluorescent adenine-analog). The fluorescent emission of this photoproduct in water, discussed later, has a quantum yield of 40%, a single-exponential

fluorescent decay of 7.5 nsec, and a pH dependence similar to that reported for 2AP.

In another example, we used the arrangement described above, with 30 uM dGMP in 30% glycerol/water with 0.1 M Tris buffer at pH 9.5. The absorption for no uv light and for 20 min illumination at 275 nm with 50 mW is shown in Fig. 4(a). The photoproduct fluorescence emission and excitation spectra are shown in Fig. 4(b). By comparison to Figs. 3(a)-3(f), it is apparent that the photoconversion at ph 9.5 Tris buffer differs very little from that in an unbuffered solution. Similar measurements at buffered solutions with pH between 6.8 and 11 indicated no strong pH dependence to the photoconversion in this range.

Other reagents were used to produce photoproducts. A 40-uM solution of dGMP in 50% 2-propanol/water, unbuffered, was illuminated with 50 mWatts of 275 nm uv light. The absorption measured at 20 min and 40 minutes of illumination are shown in Fig. 5(a), as well as the fluorescence after 40 min (Fig. 5(b)). The loss of parent dGMP is 32% and 50% at 20 min and 40 min, respectively, while the increase in absorption at 303 nm was 0.085 and 0.10. The ratio of dGMP lost to photoproduct gained (*i.e.*, the amount of 303 nm absorption) suggests the photoproduct may be decomposing with time in 2-propanol. The fluorescence properties are very similar to the glycerol/water dGMP-photoproduct, but the emission of the 2-propanol product contains a hump in the emission spectra in the 380-450 nm range, suggesting more than one emission maximum and hence a second red-shifted photoproduct.

The nucleoside guanosine (Guo), mixed in 30% glycerol/water, yielded very similar results to that found for dGMP, both in photoconversion rate and in the fluorescent properties of the product formed. This indicates that the phosphate group of the nucleotide does not play a determining role in the photochemical reaction.

Photochemistry using xanthosine 5'-monophosphate (XMP) under similar conditions yielded a fluorescent photoproduct,

with a photochemical yield smaller than dGMP, and with fluorescence excitation and emission spectra red-shifted (excitation maximum 315 nm, emission maximum 375 nm) from that of the dGMP-photoproduct. The fluorescence properties appear similar to isoinosine, a fluorescent analog of inosine, and when taken together with the similarity between the dGMP photoproduct and 2-aminopurine, suggests that photochemical modification of the parent nucleotide may involve changes at the C(6) position of the base ring structure.

Experiments were performed with dAMP and with TMP. The nucleotide dAMP exhibited a large photochemical rate in glycerol/water, ethylene glycol/water, and 2-propanol/water, and produced a photoproduct with about 50-times less fluorescence than the dGMP photoproduct. The excitation spectra peaked at 315 nm, while the emission peaked at 410 nm. The less intense emission, and the fact that the excitation spectra was red-shifted from the photoproduct absorption peaks, suggests that the dAMP-photoproduct fluorescence contains a minor fluorescent component such as a tautomer.

A TMP photoproduct was observed in the microscope setup of Fig. 2(b), but has not been quantified in the bulk arrangement of Fig. 2(a).

<u>Fluorescence Properties of the dGMP-photoproduct</u>

Spectroscopy of the dGMP-photoproduct was performed using dilute solutions of the photolyzed samples described above. The emission and excitation spectra of a solution containing 7.7 nM dGMP-photoproduct in unbuffered water is shown in Fig. 6(a). For comparison, fluorescence from a solution of 2-aminopurine, adjusted in concentration to have the same absorbance ($5x10^{-4}$) at 303 nm as the photoproduct solution, is shown in Fig. 6(b). As can be seen, the photoproduct spectra are nearly identical to the 2AP-spectra, with a small blue-shift of the excitation and emission maxima (emission wavelength of 265 nm vs. 269 nm for 2AP). A

quantitative measurement of the quantum yield of the photoproduct is 40%, based on comparison to the fluorescence intensity of 2AP (quantum yield 95% in water). The fluorescence lifetime for optically thin, unbuffered solutions of dGMP-photoproduct and 2AP are shown in Fig. 7. The lifetimes are 7.5 nsec and 9.5 nsec, respectively. Due to the similarity of the absorption and emission spectra between dGMP-photoproduct and 2AP, as well as the approximate equality of the extinction coefficients, the single-exponential lifetime for dGMP-photoproduct (7.5 nsec) suggests its quantum yield should be about 65%, based on Strickler-Berg arguments.

The pH dependence of the fluorescence intensity of the guanosine photoproduct is shown in Fig. 8. (The dGMP-product behaves similarly). As can be seen, the emission is approximately constant from pH 4.5 to 11, with some variation probably arising from the type of buffer used. The emission intensity drops to half maximum at pH 3.5 and 11.5. This dependence is in close agreement with that found by Ward for 2AP-riboside monophosphate, who reported excited-state pK values of 3.6 and 12.1. Ward,D. C., and Reich, E., "Fluorescence Studies of Nucleotides and Polynucleotides. I. Formycin, 2-aminopurine ribose, 2,6-diaminopurine riboside, and their derivatives", J. Biol. Chem. 244, 1228 (1969). Additionally, Ward's observation of a weak but red-shifted emission spectrum at low pH is also observed here for the G-photoproduct. The conclusion is that the sites of protonation for the photoproduct are very similar to those of 2APMP.

To determine the photobleaching rate of the photoproduct, the photoproduct made from the irradiated 15 uM dGMP solution (Fig. 3(e)) was diluted 30-fold in unbuffered water, and illuminated with 80 mW of 293 nm light. In the absence of N_2-purging, a bleaching rate of 23 min was obtained, while a similarly prepared sample but nitrogen-purged bleached much more slowly, on the order of several hundred minutes. For the absorbed number of photons of

$2x10^{17}$, and $2x10^{18}$, the photobleaching yields of 0.1% (no N_2 purging) and 0.01% (with N_2 purging), were obtained. Therefore, the photoconversion of dGMP to photoproduct proceeds at about 10x the rate at which the photoproduct is bleached. This indicates it is possible to get on average 10,000 photons emitted per photoproduct molecule before bleaching using a N_2-purged solution.

Discussion of Bulk Nucleotide Photochemistry

In the absence of a complete molecular structure analysis, the G-photoproduct has been characterized here by its spectroscopic properties, which as noted are remarkably similar to those of 2-aminopurine. It is worth noting that guanine and 2-aminopurine, differ only in the substitution at the C(6) carbon of the purine ring as shown in Fig. 9. As also noted above, xanthine-photoproduct and isoinosine have similar fluorescence properties, and their structures also differ only at the C(6) position as also shown in Fig. 9. This suggests that the photochemical reaction involves elimination of the keto oxygen at the 6-carbon of the purine ring. However, the generality of an elimination of a C(6) substituent following photolysis could be tested by examining the photoproducts of 2,6-diaminopurine, as well as that of isoguanosine. Both contain an amino group at C(6), which upon removal reduces these molecules to the fluorescent analogs 2AP and isoinosine, respectively.

Binding and Immobilization of Nucleotides and Photoproducts to Surfaces

The immobilization of monophosphate nucleotides at an liquid/solid interface following their sequential cleavage from a strand of DNA represents a useful method for their subsequent detection at the single molecule level. It is known that metal oxides such as aluminum oxide (alumina) preferentially binds nucleotides but not nucleosides or bases. Ramachandran, J "A new simple method for separation of Adenosine 3',5'-cyclic monophosphate from other

nucleotides and its use in the assay of adenyl cyclase", Analytical Biochemistry 43, 227 (1971); Kouni, M. H. El and Cha, S. "A simple radioisotopic assay for nucleoside kinases employing alumina for separation of nucleosides and nucleotides", Analytical Biochemistry 111, 67 (1981); Coletti-Previero, M.-A. and Previero, A. "Alumina-phosphate Complexes for Immobilization of Biomolecules", Analytical Chemistry 180, 1 (1989). The binding results from the electrostatic attraction of the negatively-charged phosphate group of a nucleotide with the positively-charged aluminum ions on the alumina surface. Examples of possible coordination of phosphate on alumina are discussed by Rajan, S.S.S. "Changes in net surface charge of hydrous alumina with phosphate adsorption", Nature 262, 45 [1976]; Rajan, S.S.S. "Adsorption of divalent phosphate on hydrous aluminum oxide", Nature 253, 434 [1975].

Other surfaces, particularly monolayer films applied by the Langmuir-Blodgett technique, can be used. For example, to bind to monophosphate nucleotides, we have used an aluminum-alkanebisphosphonate thin film such as that used to immobilize DNA as described in Xu, X.-H., Yang, H. C., Mallouk, T.E., and Bard, A. J., "Immobilization of DNA on an Aluminum (III) alkanebisphosphonate thin film with electrogenerated chemiluminescent Detection", J. Am. Chem. Soc. 116, 8386 (1994).

A simple procedure was used to make substrates with nucleotides bound to the surface. A quartz substrate, one surface of which was coated by vacuum deposition with a thin, 10-nm aluminum-oxide film, is covered with an aqueous solution containing nucleotides at a concentration of typically 50-200 nM. After a few minutes to allow the nucleotides in solution to diffuse to the surface, the quartz substrate is rinsed with water to remove any nucleotides not bound to the surface, then dried with nitrogen, and covered with either water or another solvent. Surface coverage assays using scintillation counting of ^{32}P-labeled dAMP, or fluorescence detection of dye-labeled nucleotides

(fluorescein-labeled dUTP), or fluorescent-analog nucleotides (2APTP, etheno-dAMP, dGMP-photoproduct) show that the alumina surface adsorbs up to about 10^4 nucleotides per square micron (about 1% of a monolayer), which is the apparent saturation coverage. At these coverages, dimer formation on the surface is not a significant factor, so that the photophysical properties measured will reflect the behavior of monomer nucleotides, and can be extrapolated to low surface coverage, i. e. single surface-bound nucleotides. By comparison, the nucleotide binding efficiency to a quartz surface with no alumina is down about 10,000-fold. As discussed below, alumina-bound nucleotides can remain bound for hours, with a 1/e off-rate of about 1/(7 hrs) at room temperature. The use of a buffered solution of nucleotides may or may not inhibit binding, *e.g.*, a basic glycine buffer reduces binding on alumina by greater than 10-fold, whereas a basic Tris buffer does not. But once the nucleotides are bound to the surface, changing the solution buffer does not appear to displace the nucleotides from the surface, although very acidic solutions or phosphate-buffered solutions appear to displace bound nucleotides.

We used the experimental arrangement shown in Fig. 2(b) to measure the photophysical properties of nucleotides bound to an alumina surface. Samples of surface-bound nucleotides were prepared using the procedure described above. Fig. 10(a) shows the change in fluorescence intensity with time from fluorescein-labeled dUTP bound to an alumina surface. Low excitation power is used to minimize bleaching of the fluorophore. The decay in the emission with time reflects the reduction in the number of nucleotides bound to the surface. The data can be fit assuming an exponential decay of the number of nucleotides bound. The fitted off-rate was determined to be 3.8×10^{-5} sec^{-1}, which yields a 1/e off time of about 7 hours. Similar off times were found for monophosphate nucleotides.

Having established that the nucleotides remain on the surface, we then examined the magnitude of the diffusion of

nucleotides along the surface using the Fluorescence Recovery After Photobleaching (FRAP) method: A region of the surface is illuminated with high power to bleach all of the molecules on that part of the surface. Subsequent fluorescence detection at low (non-bleaching) power then provides a means to determine the rate at which nucleotides diffuse along the surface into that region. Fig. 10(b) shows the fluorescence intensity before and after the bleaching pulse. As can be seen, after bleaching, the fluorescence intensity is reduced by 20x, and remains at this value for greater than 100 min. This constant signal is partly due to binding of nucleotides from the solution, since the solution will contain a very small amount of nucleotides previously bound but desorbed back into the solution. Surface diffusion into the bleached area of the sample is very slow, estimated to be less than 10^{-10} cm^2/sec. Similar rates were found for monophosphate nucleotides. The conclusion drawn from this work is that once bound on alumina, nucleotides remain immobile for hours at room temperature (and presumably much longer at low temperatures). This arrangement then allows for the detection of single nucleotides on a surface.

Fig. 11 shows the fluorescence lifetime of surface-bound 2-aminopurine triphosphate (2APTP) and the bound nucleotides from a solution containing dGMP-photoproduct (taken from the photolyszed bulk solution of Fig. 3(e)). The lifetimes were measured using unbuffered water as the solution in contact with the surface-bound nucleotides. The lifetimes are best fit to a two-component decay, as opposed to the lifetimes measured in solution, which yielded single-exponential decays. The slow component (6-7 nsec) is approximately equal to that expected based on the solution decay, while about 16% of the bound nucleotides exhibit a short lifetime of about 1.5 nsec. Although a distribution of lifetimes can arise for randomly-oriented fluorophores at the interface between media with differing dielectric constants, calculations indicate this is a small effect, and cannot account for the short-lifetime component. More likely, the different decay times

reflect different environments of the surface-bound nucleotides. Alumina is known to have acidic and basic binding sites. Thomas, J. K., "Physical aspects of photochemistry and radiation chemistry of molecules adsorbed on SiO2, γ-Al_2O_3, zeolites, and clays", Chem. Rev. 93, 301 (1993). This heterogeneity may be responsible for the two-time fluorescence decay. When overcoated with a pH 5 acetic acid-sodium acetate buffer, the fluorescence decay can be fit well with a single exponential, and we estimate that the fraction of bound nucleotides with a short-lifetime decay is reduced to less than 5%. In addition to solution pH, various methods to further enhance homogeneous binding sites can be employed, such as capping the acidic sites prior to nucleotide binding. Langmuir-Blodgett films may also be used to provide more homogeneous binding. The conclusion drawn from these lifetime measurements are that the quantum yield for nucleotides on an alumina surface is essentially that found in solution, while a small portion (5-15%)of the bound nucleotides have a quantum yield about 4x smaller than that in solution.

The measurements and results found here for surface-bound nucleotides indicate that in all important respects, the fluorescence properties are essentially unchanged from those found in solution. There is no indication of undesirable processes such as charge-or-energy-transfer from the nucleotide to the surface. These findings agree with the statement in the review by Thomas, J. K., "Physical aspects of photochemistry and radiation chemistry of molecules adsorbed on SiO2, γ-Al_2O_3, zeolites, and clays", Chem. Rev. 93, 301 (1993) that the photochemistry (and presumably the photophysics) on surfaces are reminiscent of reactions in polar homogeneous solution (*e.g.*, water).

Detection of Single Photoproduct Nucleotides Bound to a Surface

The ultimate measure of detection sensitivity is the detection of uv-excited, uv-fluorescent single molecules. A

key issue is the reduction of background fluorescence from the medium surrounding the single molecule of interest. In our experimental arrangement of Fig. 2(b), low background fluorescence is achieved by the combined use of (1) high-purity quartz substrates (Corning 7904, polished to better than 5-10 scratch-dig and sold by CVI, Inc.), (2) very thin films of alumina (aluminum oxide will fluoresce under uv illumination, so only a minimum film thickness to insure coverage of the quartz substrate is necessary), cleaned in a hot acid/peroxide bath prior to use for immobilizing a molecule, (3) evanescent-wave excitation so that only a region within about 0.03 microns of the alumina/quartz surface is excited, and so the solvent is minimally excited, (4) ultrapure water and solvents free of fluorescent impurities, (5) a 100x, oil immersion quartz objective, the solid angle of which collects a calculated 20% of the total light emitted by a surface-bound molecule, and (6) optical filters to maximize the rejection of background light outside the emission bandwidth of the molecule of interest.

The following method was used to detect single dGMP-photoproduct nucleotides. An alumina-coated quartz substrate was first coated with dGMP photoproduct at a coverage of about 1000 molecules per square micron. This sample was then placed in a glove bag, covered with a drop of unbuffered water, and purged with nitrogen for 30 minutes. While still in the nitrogen-filled glove bag, the sample was then covered with a quartz coverslip, using a thin elastomer (PDMS) to seal the coverslip on the alumina/quartz substrate. In this way, we could make a sealed cartridge containing nitrogen-purged water (water thickness about 20 microns) overcoating the nucleotides bound to the alumina/quartz substrate. This "sandwich"-like cartridge was then placed on the microscope stage for evanescent excitation at 293 nm, with collection by an oil-immersion, 100x objective (1.2 NA) through the coverslip. For this objective, the resolution is 0.18 microns, and the collection/detection efficiency about 2.5%. The fluorescence from the sample surface was imaged onto a

CCD camera. When using a 100x objective, each camera pixel (size of 25 microns) images a 0.25 micron by 0.25 micron area of the sample. Thus, if there is a coverage of 1000 molecules per square micron on the sample, each camera pixel records fluorescence from an average of about 67 molecules.

Once on the microscope stage, a small region on the alumina/quartz was first illuminated with high intensity uv light (293 nm), to bleach all of the nucleotides in that region. The uv intensity was then turned down, and fluorescence images taken of the sample. Since the water solution above the surface will always contain a very small concentration of photoproduct nucleotides that have desorbed from other unbleached regions of the surface, these nucleotides will continually re-bind to the surface, and hence some will rebind to the bleached region. Therefore, a fluorescence image of this bleached region of the sample can show single nucleotides binding to the alumina surface at random locations.

Fig. 12(a) shows one series of fluorescent images taken with 0.5 mWatts of 293 nm power (an intensity of about 50 W/cm^2). Each image, or frame, collects light for 2 sec., and there is a dead time between frames of about 1/2 sec during which the data is read-out and the camera is off. These images show that single bright pixels with signals well above the noise appear for typically one to several frames, and then disappear. In Fig. 12(b) we show a linecut through the images in Fig. 12(a) for several frames, to shown more clearly the appearance and disappearance of single nucleotide molecules. We believe these bright pixels represent single photoproduct nucleotides binding to the alumina, which after a brief period, undergo bleaching. We base this argument on the facts that (1) if the same sample preparation procedure is used but no nucleotides are added, the fluorescence images can show similar bright pixels but at a much reduced frequency of occurrence, (2) the fluorescence signal observed, typically 30-40 counts over the background, is close to the signal expected from a single G-photoproduct

nucleotide, based on the excitation intensity used and the extinction coefficient of the photoproduct, (3) a typical number of photons detected from a bright pixel (i. e. a molecule) before 'bleaching' is about 100-200 photons, roughly in agreement with the bleaching rate expected for the photoproduct, and (4) the optical filters used only pass wavelengths from 330 nm to 395 nm, and therefore limit the possibility that these are impurity molecules. Further work is underway to incorporate triplet quenchers to further reduce the photobleaching of the nucleotides, resulting in more photons per single molecule than obtained here. Under these conditions, it would be desirable to perform spectroscopy on single molecules (Trautman, J. K., Macklin, J. J., Brus, L. E., and Betzig, E., Nature 369, 40 (1994)); Macklin, J. J., Trautman, J. K., Harris, T. D., and Brus, L. E., "Imaging and time-resolved spectroscopy of single molecules at an interface", Science 272, 255 (1996) at higher resolution than that used here with crude optical filters. From this work, we conclude that single nucleotide detection using the dGMP photoproduct can be accomplished, and with improvements in reduction of background and increased photostability, can be a reliable scheme for detecting single nucleotides.

Photoconversion of Nucleotides immobilized on a Surface

Measurements on photochemical conversion of surface-bound dGMP to fluorescent products were carried out using the experimental arrangement shown in Fig. 2(b), with both epi-illumination as well as with evanescent excitation, at 260 nm. dGMP was deposited on an alumina-film/quartz substrate, as described above, to an approximate surface coverage of about $10^4/um^2$. A 30% glycerol/water drop was placed over the surface, and the whole sample area purged with nitrogen for 30 min. The sample was then irradiated with uv light. Based on the spectrum and intensity of the fluorescence in the bandwidth of the photoproduct under 260 nm illumination, these measurements indicated about 10% of the parent dGMP

nucleotide was converted to a fluorescent product. (Note in this arrangement, the 260 nm light both initiates the photochemistry, and is used to excite the photoproduct fluorescence).

To test if dGMP in solution and near (but not bound to) a surface can be converted, the alumina/quartz substrate was replaced with just quartz, and covered with a drop of 30 uM dGMP in water. For the same evanescent-wave excitation, significant photoproduct was observed after purging this arrangement with nitrogen, indicating that the photochemical reaction proceeds very close (within 10 nm) to the surface. Taken together with our result that photoproducts made in solution and then deposited on alumina exhibit no significant degradation in fluorescence properties, the lower photoproduct conversion may arise in the molecular interaction between nucleotide and reagent. For example, molecular orientation of the bound nucleotide may hinder the necessary approach by the reagent molecules, thus preventing a necessary orientation and proximity. This can be tested using a less rigid (more fluid-like, but equally strong) binding layer, to allow the nucleotides greater rotation or conformational freedom. Additional information on conversion yield for alumina are under way, with initial tests to understand the role of solvent pH on the photochemistry.

Alternate Embodiments

The solution photochemistry described above requires an reagent that can contact the nucleotide, and we have demonstrated examples of such a reagent in glycerol, isopropanol, and ethylene glycol. The active reagent cannot just be water, since photolysis of a solution containing only water and dGMP does not result in a fluorescent product. In addition, unless the solution is purged with nitrogen or another gas to remove dissolved oxygen, the photochemical yield is very low. So an active component of the photochemistry is the replacement of dissolved oxygen with another gas such as nitrogen or argon.

As a general rule, photochemical reactions precede from the triplet state of a molecule. Here, a nucleotide absorbs light and as a result occasionally forms a triplet state, a state that is chemically reactive and usually metastable (lifetime from 10 usec to 1 msec in solution). For chemical reactions, a long-lived triplet state is desirable to increase the time for encounters with reagent molecules. This accounts for our need to purge the solution of dissolved oxygen, since oxygen is a well-known triplet quencher that shortens the triplet lifetime. Other methods to modify the triplet state dynamics, for example, by increasing the intersystem-crossing rate, should improve the photochemical quantum yield.

Another general rule is that photochemistry from reagents to products often takes place via radical intermediates, formed initially by hydrogen- or electron-abstraction from a donor reagent molecule. The alcohols we have used in our work are known to be good hydrogen-atom donors. Useful reagents for photochemistry could be chosen from a list of hydrogen-atom donating molecules that includes alcohol-, sugar-, amine-, hydrocarbon- containing compounds, and this list includes nucleic acid components as well. Since the photochemistry may proceed where an active reagent absorbs the uv light, followed by the abstraction of a hydrogen atom from nucleotide, reagents that are good hydrogen-atom acceptors, such as aromatic ketones like benzophenone, may also be helpful. The principal issue in the choice of the reagent is the specificity of the photochemical reaction to produce a fluorescent product of a nucleotide, while additionally that product should not undergo photochemical reactions itself.

The photochemical reaction can be obtained with uv-illumination at a wavelength outside of the absorption band of the nucleotide by use of a photosensitizer. The reaction solution would consist of nucleotides, active reagent and sensitizer. Ultraviolet light would be absorbed by the sensitizer, which either directly formed radicals of the

reagent or nucleotides, or by energy transfer excited the nucleotide triplet state, which then formed radicals. Sensitizers are well-known in photochemistry, an example of which is acetone.

For detection and photochemistry of nucleotides immobilized on a surface, we used one simple method of binding that involved the electrostatic attraction between the negative phosphate of the nucleotide and the positive ion of a metal oxide, alumina. Other surfaces may be used with equally good binding properties, but which also allow the nucleotides greater freedom of motion. In general, a metal-ion ligand connected by a linker that is covalently attached to a substrate surface such as quartz could provide a suitable binding site for nucleotides. The length of the linker should be chosen to allow the bound nucleotide rotational and conformational motion, which is desirable to increase the photochemical reaction rate.

The binding is not limited to electrostatic forces due to metal ions. The nucleotides could be immobilized on a surface by application of an electric field, either externally applied or due to charged particles co-located on a surface. Another advantageous binding arrangement would be the use of enzymes or proteins covalently attached to a surface, that either generally or specifically bind nucleotides with high affinity. Any binding layer in all cases would likely be as thin as practical, but does not need to be a continuous film, and may intentionally or unintentionally consist of strips or islands of binding material, so chosen to advantageously minimize diffusion of the nucleotides. The thin binding layer on a substrate used to immobilize the nucleotides may also act itself as a reactive solvent, or otherwise participate in the photochemistry.

The substrate containing the surface-immobilized nucleotides can be processed to further decrease the off-rate and limit diffusion of the bound nucleotides. For example, a solution containing "blocking" molecules that bind to the

alumina can be spread over the surface. A blocking molecule could be riboside-monophosphate (a nucleotide without a base) that binds up the remaining available sites on the alumina surface, without displacing the nucleotides. The blocking molecules act to (1) prevent binding of reagent molecules in subsequent processing steps, and (2) further reduce the diffusion of nucleotides along the surface (by analogy to the restricted movement of cars in a filled parking lot). Once the excess alumina sites are tied up with blocking molecules, this solution can be washed off, and replaced with another. The sample is then ready for the process of fluorescence enhancement and detection of the surface bound nucleotides. Or for storage purposes, a non-polar solvent can be placed over the binding surface, which we have found can reduce the nucleotide off rate five-fold. Additionally the substrate can be stored at low temperature indefinitely, with no displacement of the nucleotides, until needed for the detection process.

In an advantageous arrangement of the uv illumination to perform photochemistry on surface-bound nucleotides, the polarization of the light can be changed during the course of the photochemistry, or the sample containing the nucleotides reoriented, for the purpose of providing equal amounts of light energy along the three possible directions in space, insuring that every molecule receives equal excitation, independent of its orientation. The geometry for the photochemistry of the nucleotides can be arranged so that either a real (i.e. propagating) uv electric field or an evanescent (i.e. exponentially-damped, non-propagating) uv electric field is used in the photochemistry. Both fields produce real absorption of light by molecules and therefore result in photochemistry, however, an evanescent field can be advantageously used to excite only the nucleotides and/or the reactive solvent very close to the nucleotides. When the photochemistry or uv illumination depletes or degrades the reactive solvent, fresh solvent

molecules can diffuse into the illuminated region of the nucleotides allowing the photochemistry to proceed.

In our experiments for detecting nucleotides on a surface, we used an evanescent-wave geometry for exciting the nucleotides, and full-field imaging of the surface florescence onto a CCD camera to detect the nucleotides. The detection arrangement described above could be modified to incorporate either a confocal detection scheme, or a confocal excitation/detection scheme, where the uv excitation is focused to a small spot on the sample, and all the emission from the sample at that spot, after suitable optical filters, is sent to a single channel detector, for example, a MCP detector or an avalanche-photodiode. One advantage of the confocal single channel approach is that the fluorescence decay at each spot on the sample can be analyzed in software to yield the presence and identity of a nucleotide based on fluorescence decay time, rather than just the fluorescence intensity. For this arrangement, the sample is rastered so that all spots on the sample are probed. This detection scheme can be extended to incorporate a slit confocal geometry, which is intermediate between our full-field geometry and a single point confocal geometry.

Although we have discussed the uv-illumination for photochemistry separately from that used for detection, we have experimentally shown that these two steps can be combined into one illumination step, for example with dGMP using 280 nm light. This nearly simultaneous illumination can be done best when the conditions for optimizing the photoconversion rate are the same as those for optimizing the fluorescence yield and photostability of the photoproduct. More broadly, two uv beams of different wavelengths and power may be used for nearly simultaneous photoconversion and detection in order to optimally perform the conversion process and the detection process.

What is claimed:

1. A method for identifying guanine nucleotides in a solution of nucleotides comprising the steps of:

contacting the nucleotides with a reagent;

illuminating the nucleotides and the reagent to selectively convert the guanine nucleotides to guanine products having enhanced fluorescence;

illuminating the solution with electromagnetic radiation to cause the guanine products to fluoresce; and

detecting the fluorescence of the guanine products.

2. The method of claim 1 further comprising the step of affixing the guanine nucleotide to a substrate.

3. The method of claim 1 further comprising the step of affixing the guanine product to a substrate prior to detecting its fluorescence.

4. A method for identifying adenine nucleotides in a solution of nucleotides comprising the steps of:

contacting the nucleotides with a reagent;

illuminating the nucleotides and the reagent to selectively convert the adenine nucleotides to adenine products having enhanced fluorescence;

illuminating the solution with electromagnetic radiation to cause the adenine products to fluoresce; and

detecting the fluorescence of the adenine products.

5. The method of claim 4 further comprising the step of affixing the adenine nucleotide to a substrate.

6. The method of claim 4 further comprising the step of affixing the adenine product to a substrate prior to detecting its fluorescence.

7. A method for identifying cytosine nucleotides in a solution of nucleotides comprising the steps of:

contacting the nucleotides with a reagent;

illuminating the nucleotides and the reagent to selectively convert the cytosine nucleotides to cytosine products having enhanced fluorescence;

illuminating the solution with electromagnetic radiation to cause the cytosine products to fluoresce; and

detecting the fluorescence of the cytosine products.

8. The method of claim 7 further comprising the step of affixing the cytosine nucleotide to a substrate.

9. The method of claim 7 further comprising the step of affixing the cytosine product to a substrate prior to detecting its fluorescence.

10. A method for identifying thymine nucleotides in a solution of nucleotides comprising the steps of:

contacting the nucleotides with a reagent;

illuminating the nucleotides and the reagent to selectively convert the thymine nucleotides to thymine products having enhanced fluorescence;

illuminating the solution with electromagnetic radiation to cause the thymine products to fluoresce; and

detecting the fluorescence of the thymine products.

11. The method of claim 10 further comprising the step of affixing the thymine nucleotide to a substrate.

12. The method of claim 10 further comprising the step of affixing the thymine product to a substrate prior to detecting its fluorescence.

13. A method for identifying at least two different nucleotides in a solution of nucleotides comprising the steps of:

contacting the nucleotides with reagents;

illuminating the nucleotides and the reagents to selectively convert at least two of the different nucleotides to different nucleotide products having enhanced fluorescence;

illuminating the solution with electromagnetic radiation to cause the nucleotide products to fluoresce; and

separatively detecting the fluorescence of each of the different nucleotide products.

14. The method of claim 13 further comprising the step of affixing the nucleotides to a substrate.

15. The method of claim 13 further comprising the step of affixing the nucleotide products to a substrate prior to detecting their fluorescence.

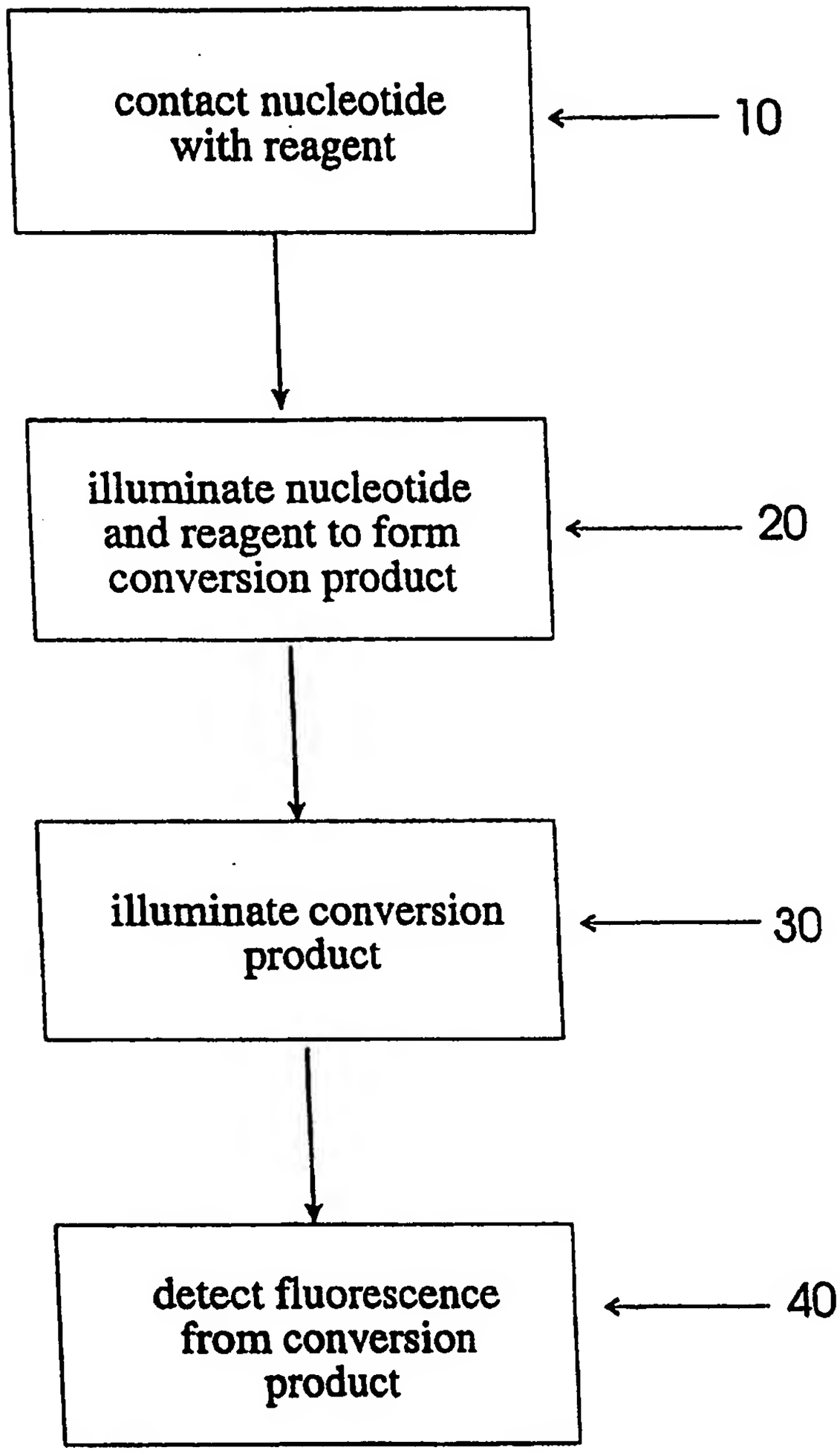

contact nucleotide with reagent
10
illuminate nucleotide and reagent to form conversion product
20
illuminate conversion product
30
detect fluorescence from conversion product
40

FIG. 1

Fig. 2(a)

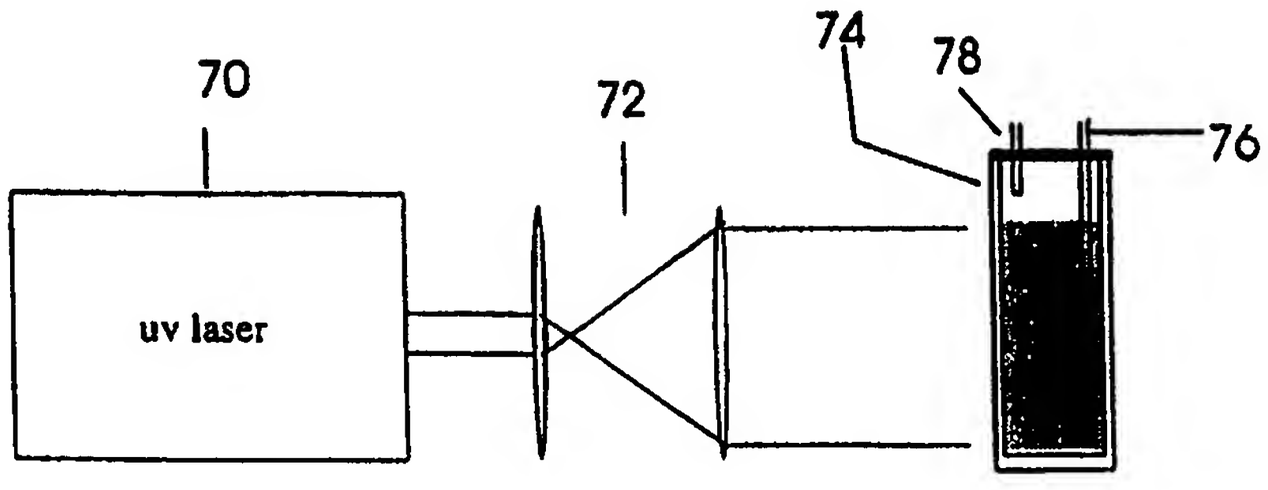
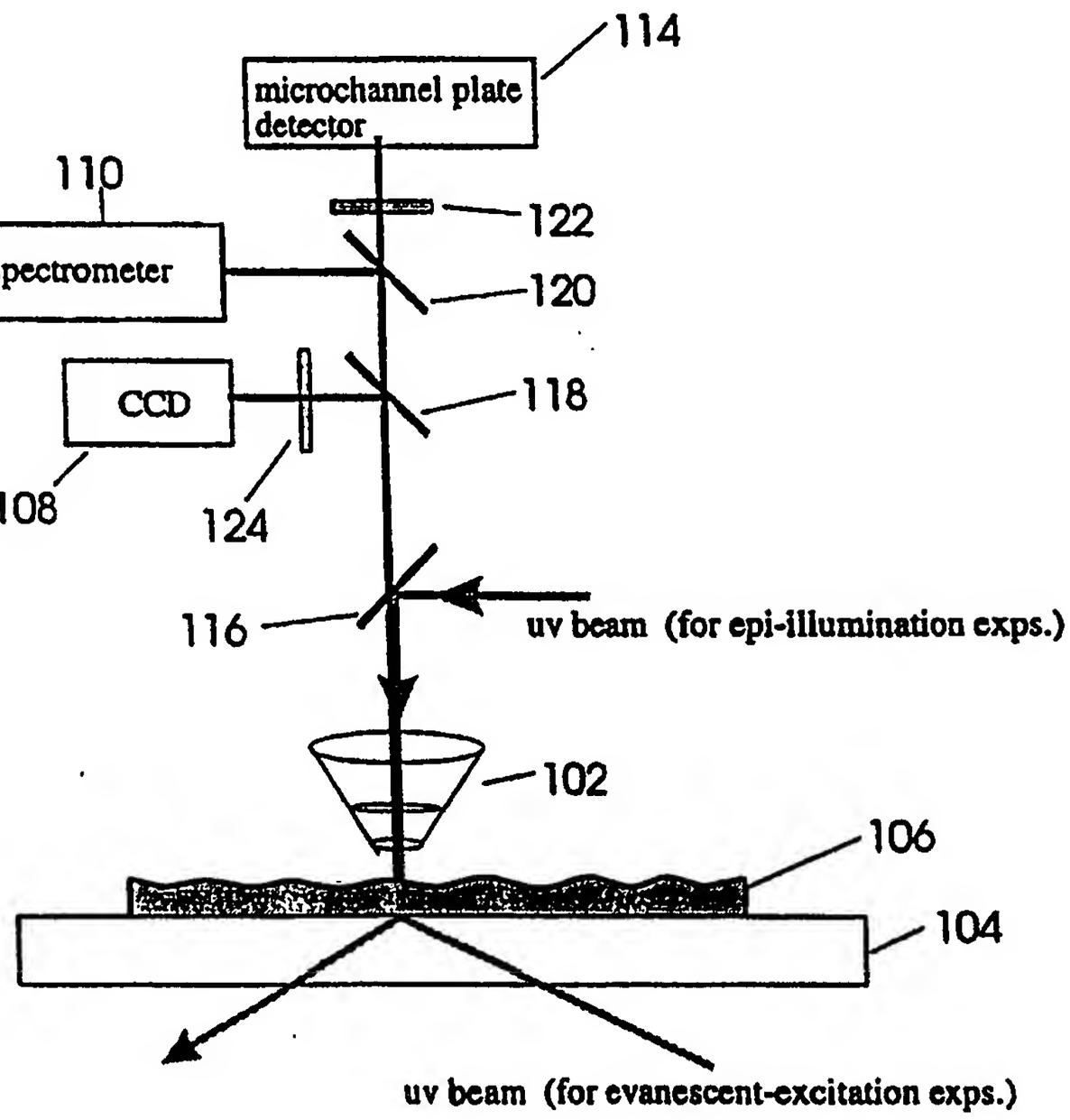

70
72
74
78
76
uv laser

Fig. 2(b)


114
microchannel plate detector
112
110
CCD
spectrometer
122
120
CCD
118
108
124
116
uv beam (for epi-illumination exps.)
102
106
104
uv beam (for evanescent-excitation exps.)

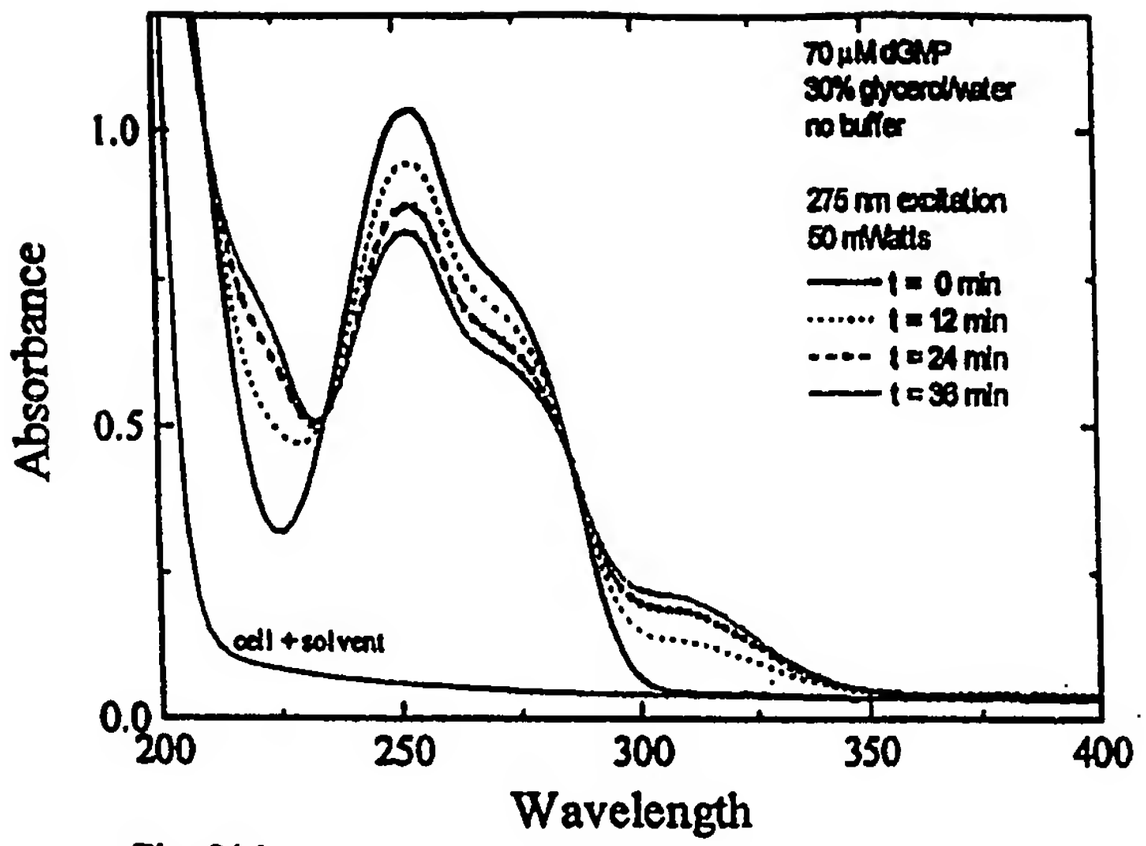

70 µM dGMP
30% glycerol/water
no buffer
275 nm excitation
50 mWatts
t = 0 min
t = 12 min
t = 24 min
t = 36 min
cell + solvent
Absorbance
1.0
0.5
0.0
200
250
300
350
400
Wavelength

Fig. 3(a)

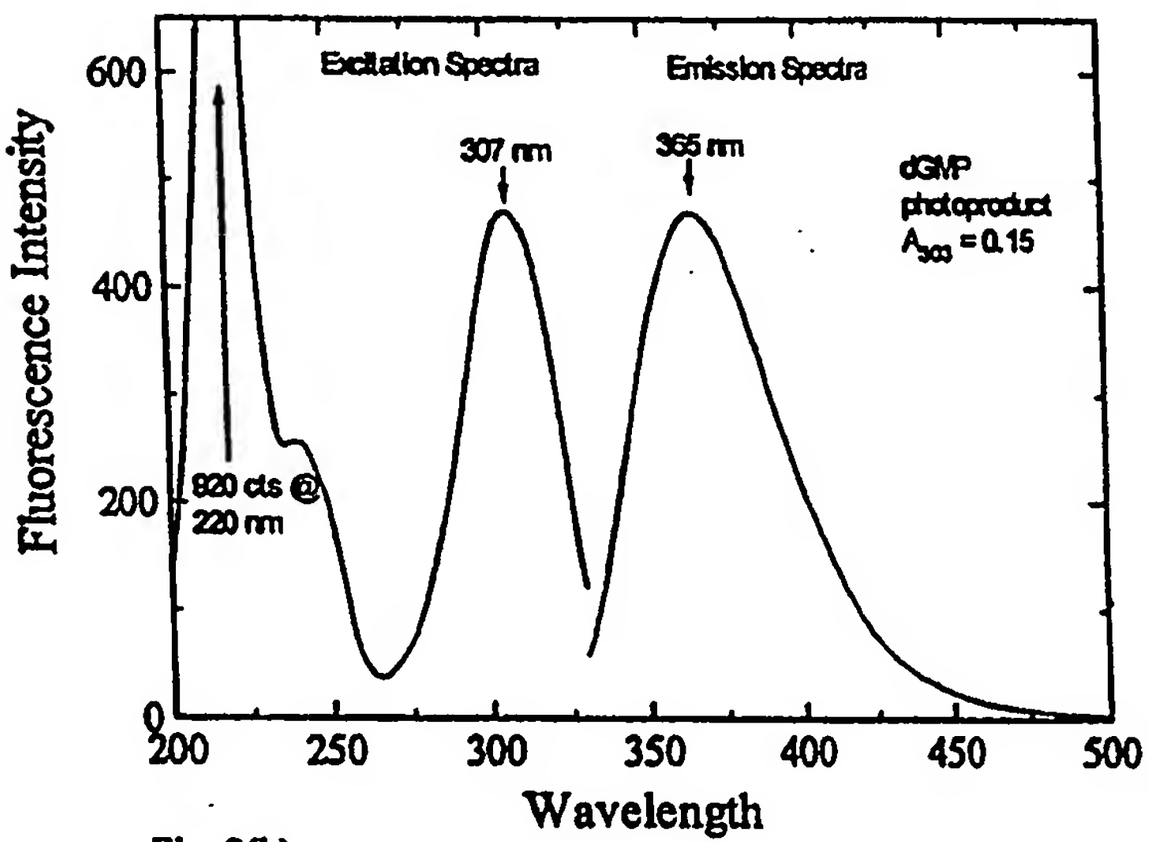

Excitation Spectra
Emission Spectra
307 nm
365 nm
dGMP
photoproduct
A_{365} = 0.15
820 cts @
220 nm
Fluorescence Intensity
600
400
200
0
200
250
300
350
400
450
500
Wavelength

Fig. 3(b)

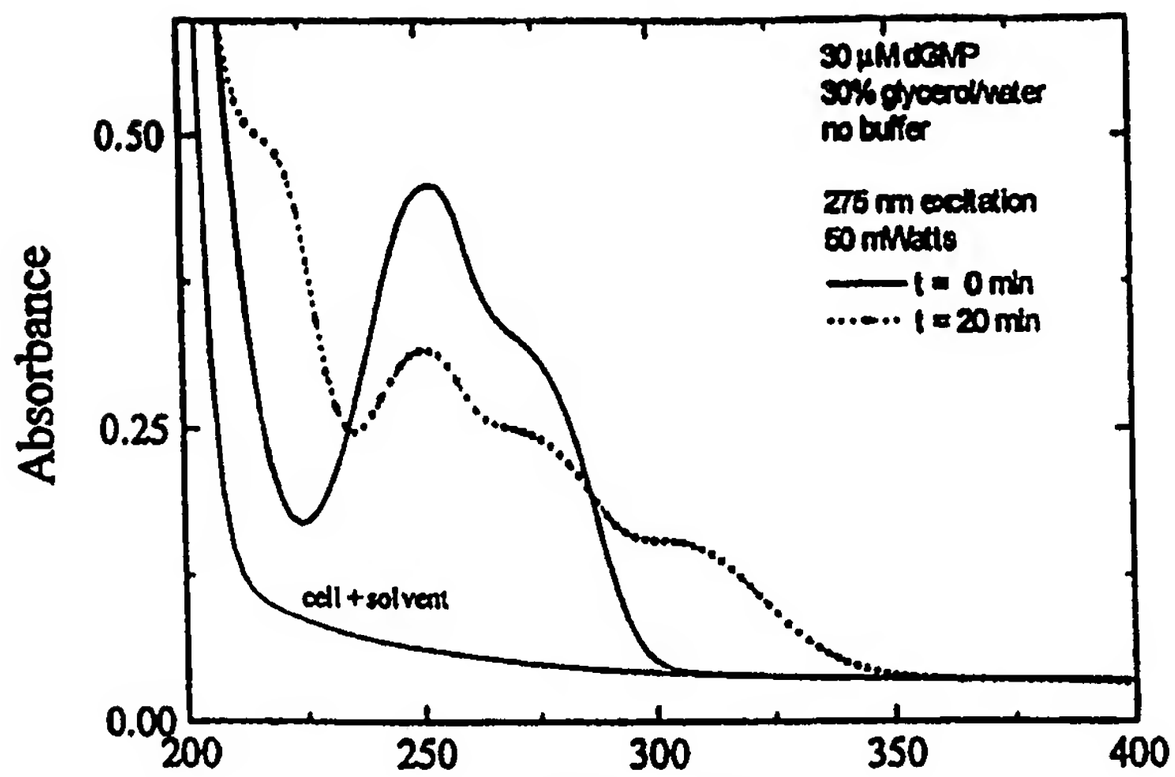

30 µM dGMP
30% glycerol/water
no buffer
275 nm excitation
50 mWatts
t = 0 min
t = 20 min
cell + solvent
Absorbance
0.00
0.25
0.50
200
250
300
350
400
Wavelength

Fig. 3(c)

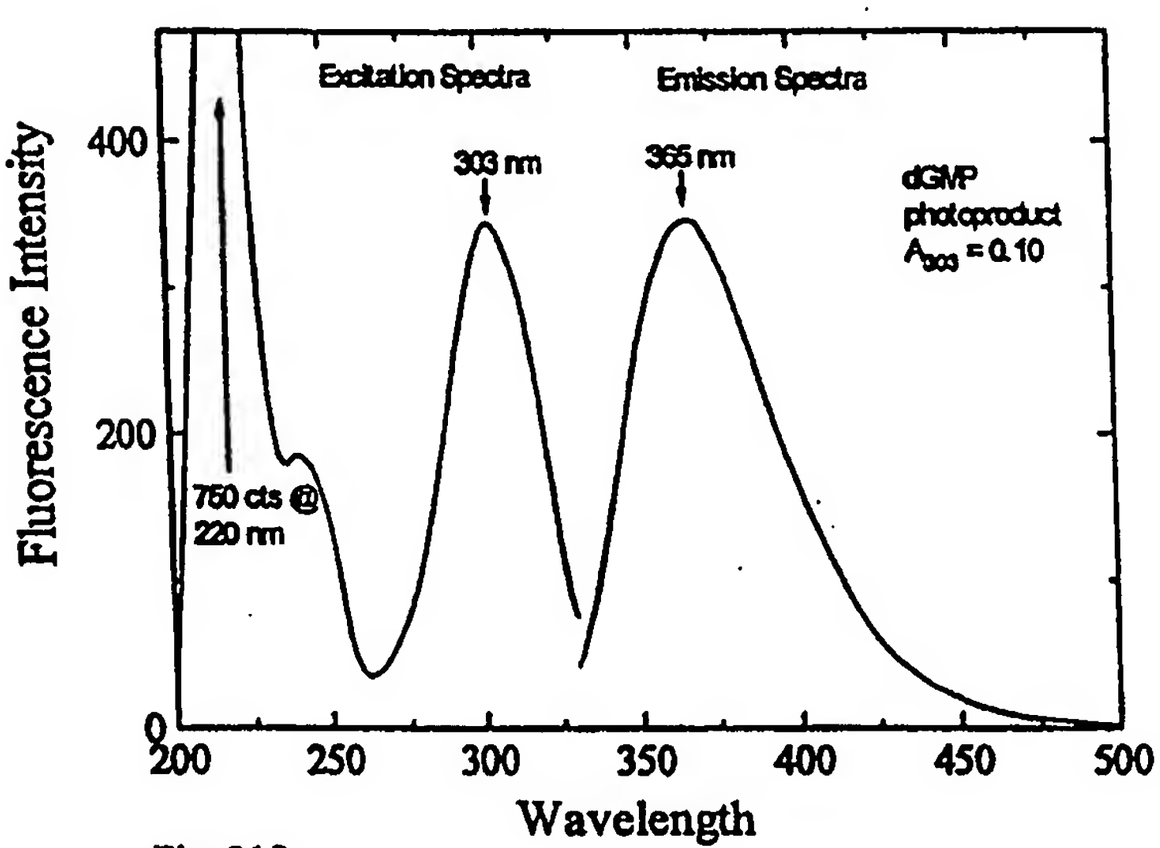

Excitation Spectra
Emission Spectra
303 nm
365 nm
dGMP
photoproduct
A300 = 0.10
760 cts @
220 nm
Fluorescence Intensity
0
200
400
200
250
300
350
400
450
500
Wavelength

Fig. 3(d)

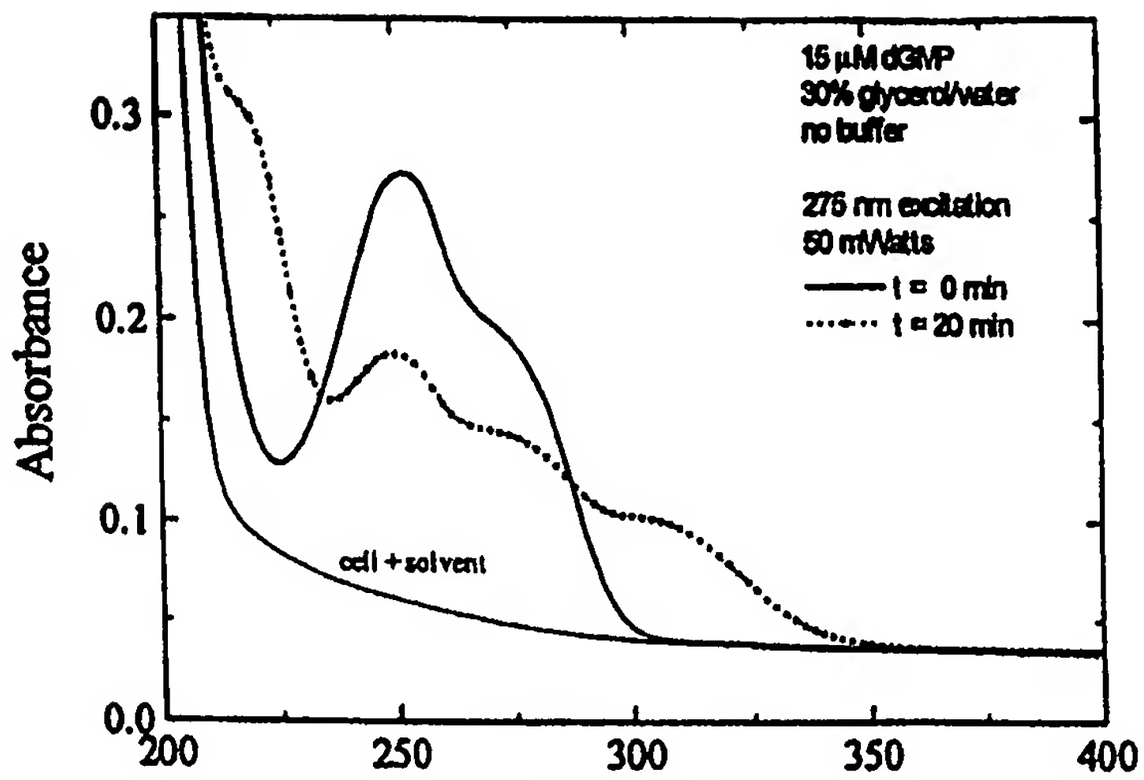

15 µM dGMP
30% glycerol/water
no buffer
275 nm excitation
50 mWatts
t = 0 min
t = 20 min
cell + solvent
Absorbance
0.0
0.1
0.2
0.3
200
250
300
350
400
Wavelength

Fig. 3(e)

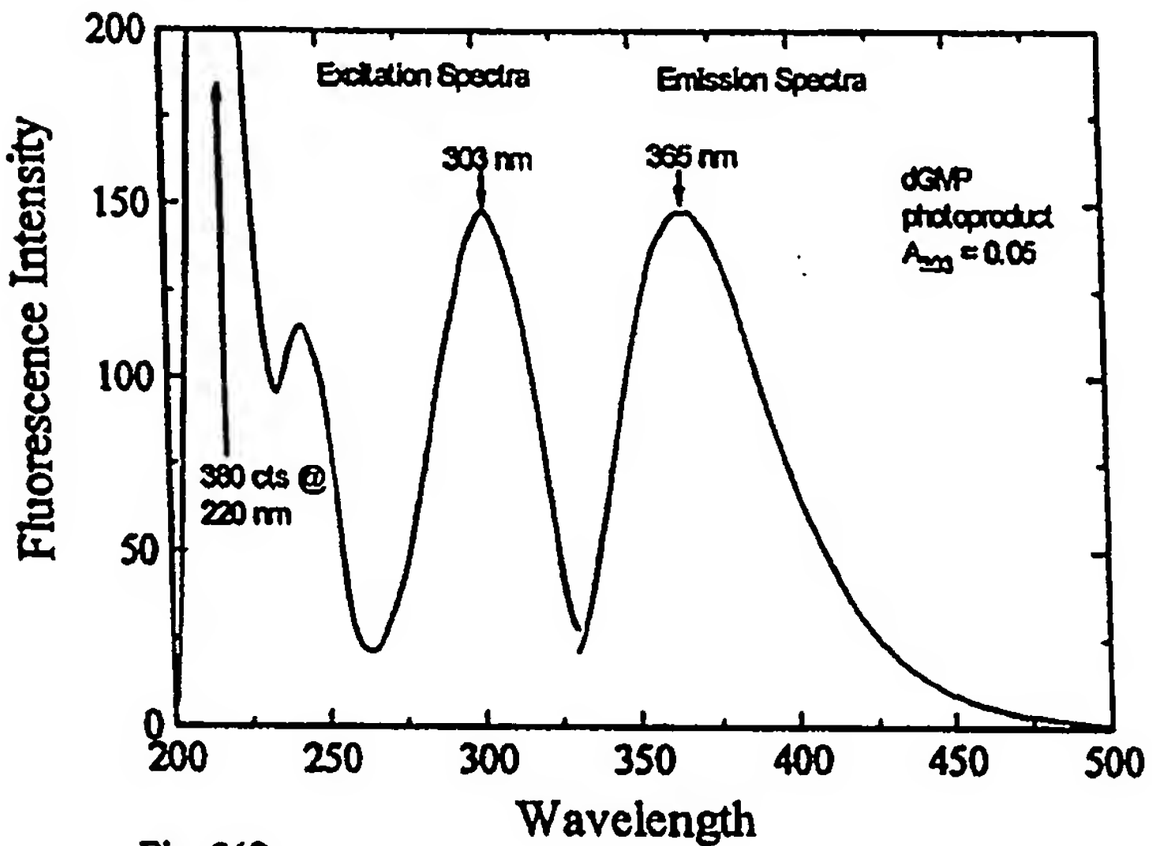

Excitation Spectra
Emission Spectra
303 nm
365 nm
dGMP
photoproduct
A_{220} = 0.05
380 cts @
220 nm
Fluorescence Intensity
0
50
100
150
200
200
250
300
350
400
450
500
Wavelength

Fig. 3(f)

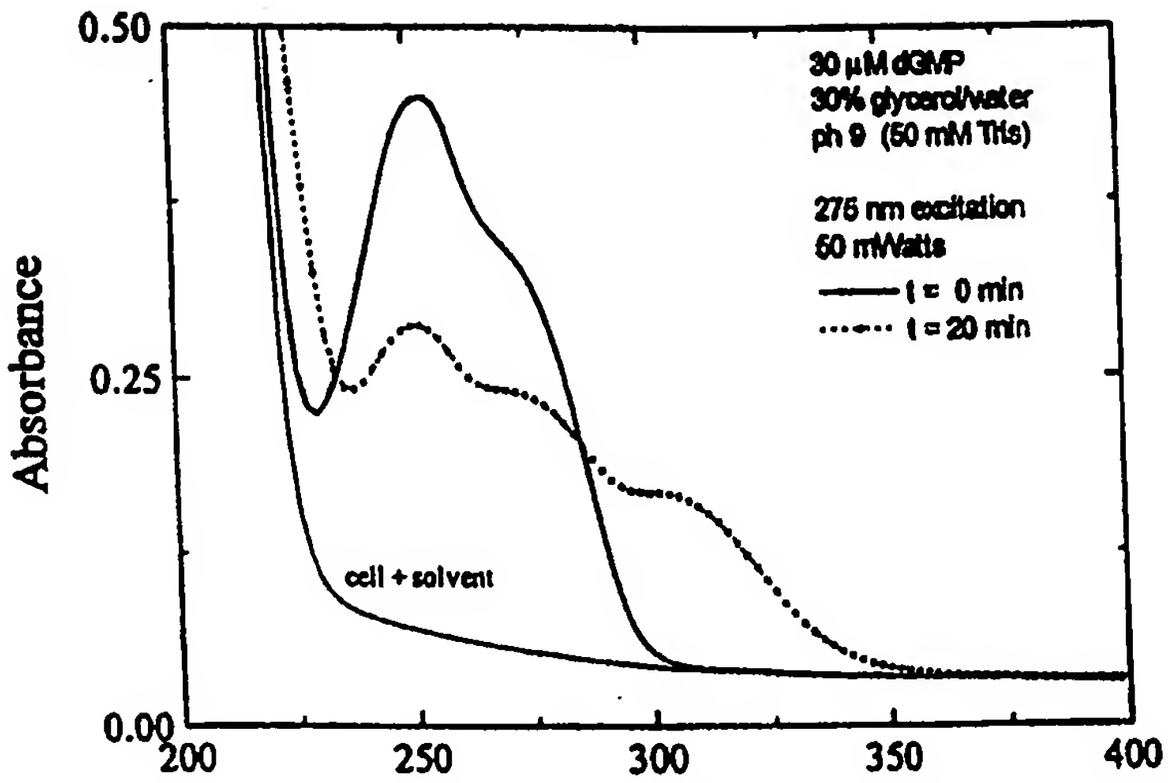

0.50
0.25
0.00
Absorbance
30 µM dGMP
30% glycerol/water
ph 9 (50 mM Tris)
275 nm excitation
50 mWatts
t = 0 min
t = 20 min
cell + solvent
200
250
300
350
400
Wavelength

Fig. 4(a)

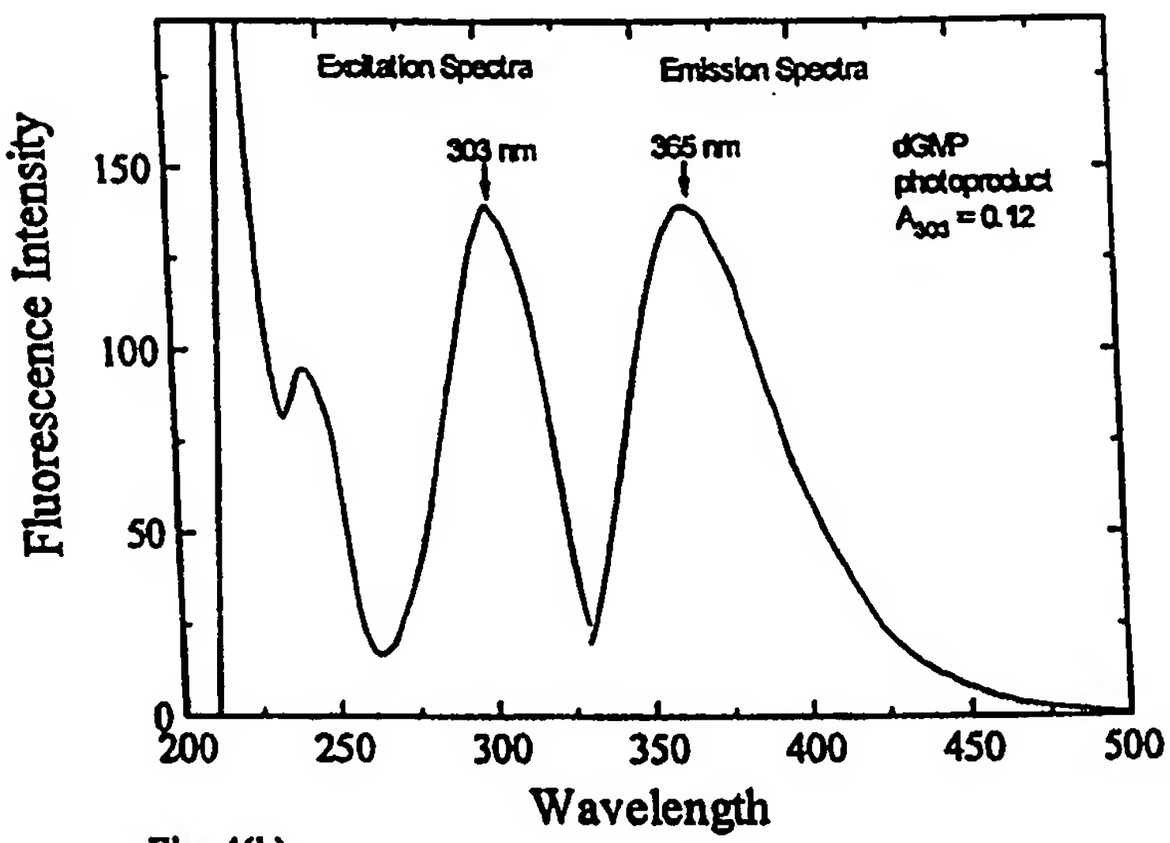

Excitation Spectra
Emission Spectra
303 nm
365 nm
dGMP
photoproduct
A_{303} = 0.12
Fluorescence Intensity
150
100
50
0
200
250
300
350
400
450
500
Wavelength

Fig. 4(b)

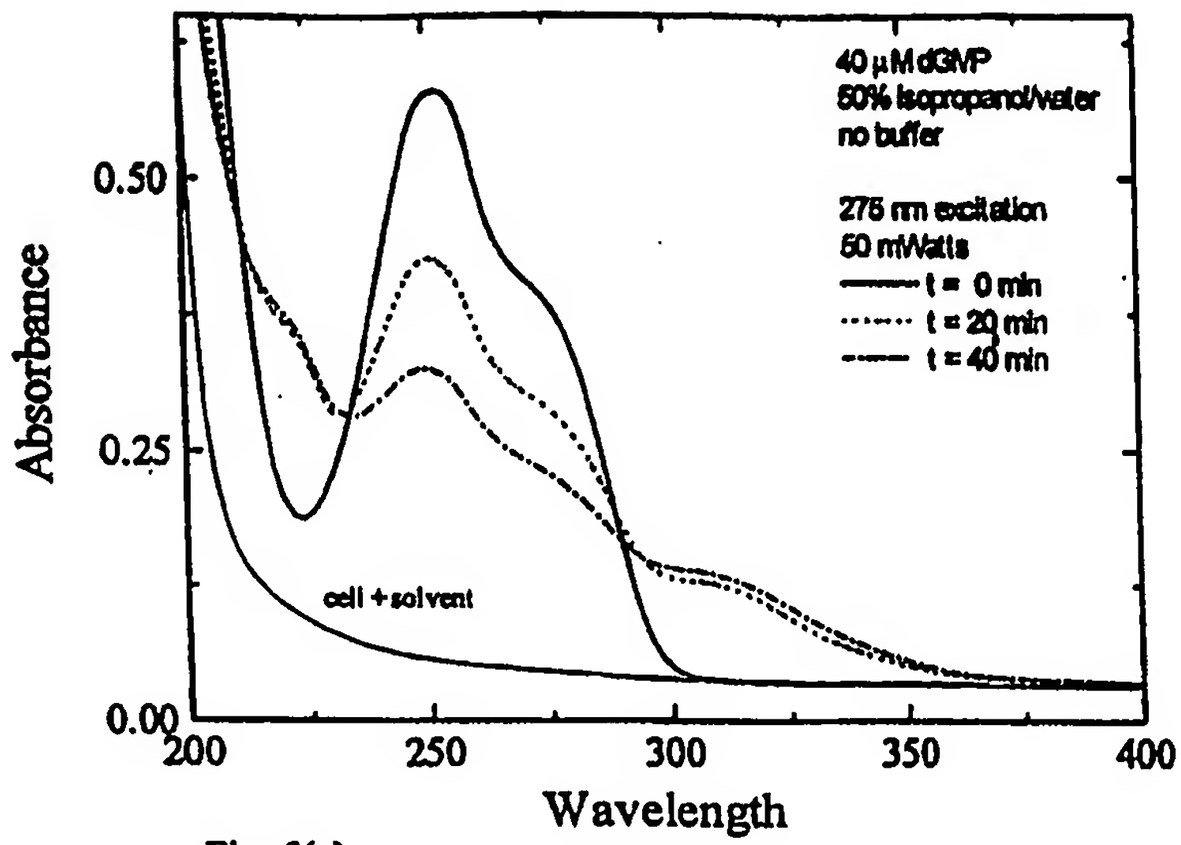

40 µM dGMP
50% Isopropanol/water
no buffer
275 nm excitation
50 mWatts
t = 0 min
t = 20 min
t = 40 min
cell + solvent
Absorbance
0.00
0.25
0.50
200
250
300
350
400
Wavelength

Fig. 5(a)

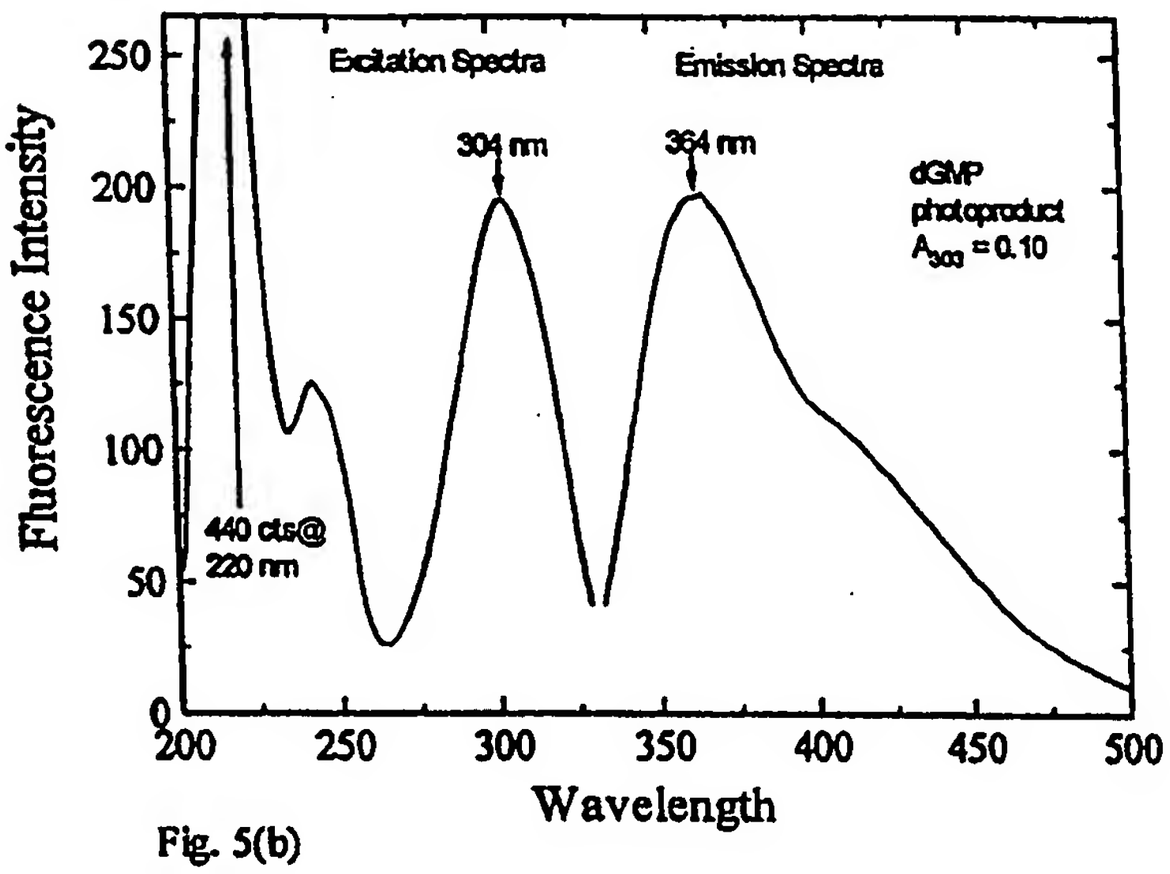

Excitation Spectra
Emission Spectra
304 nm
364 nm
dGMP
photoproduct
A300 = 0.10
440 cts@
220 nm
Fluorescence Intensity
0
50
100
150
200
250
200
250
300
350
400
450
500
Wavelength

Fig. 5(b)

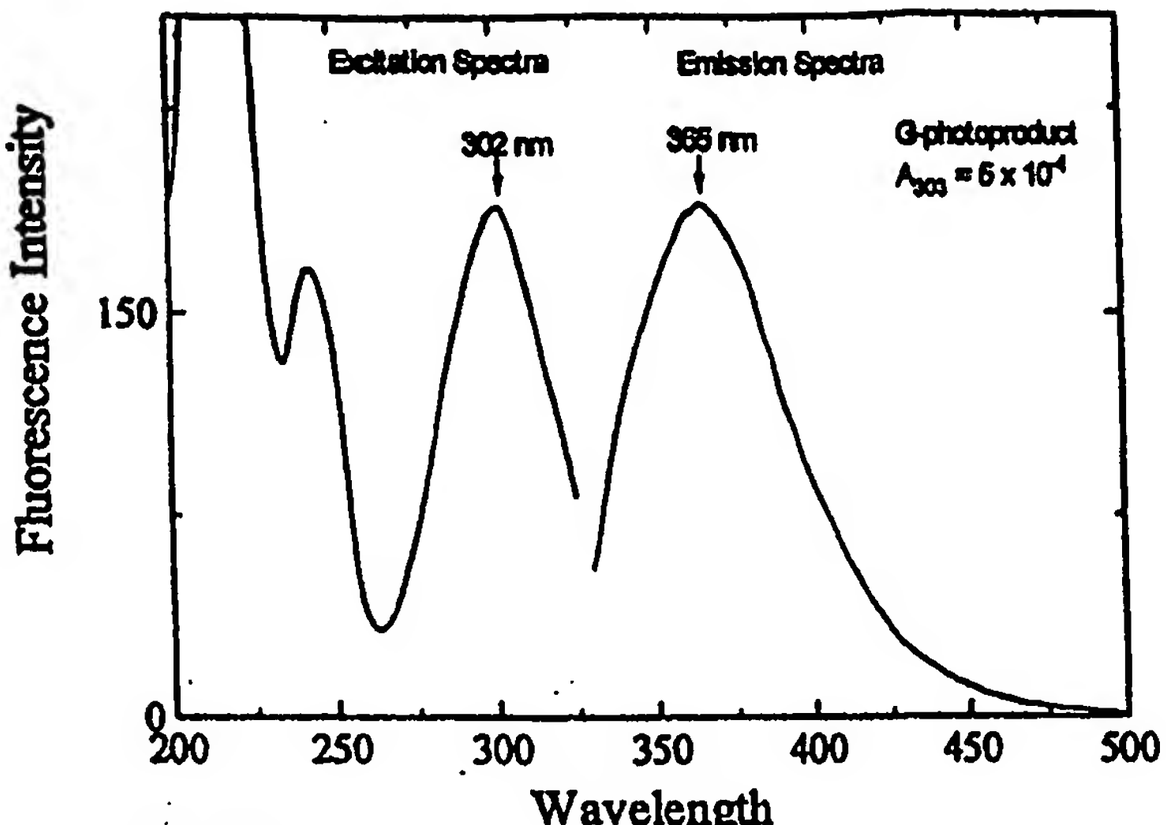

Excitation Spectra
Emission Spectra
302 nm
365 nm
G-photoproduct
$A_{303} = 6 \times 10^{-4}$
Fluorescence Intensity
150
0
200
250
300
350
400
450
500
Wavelength

Fig. 6(a)

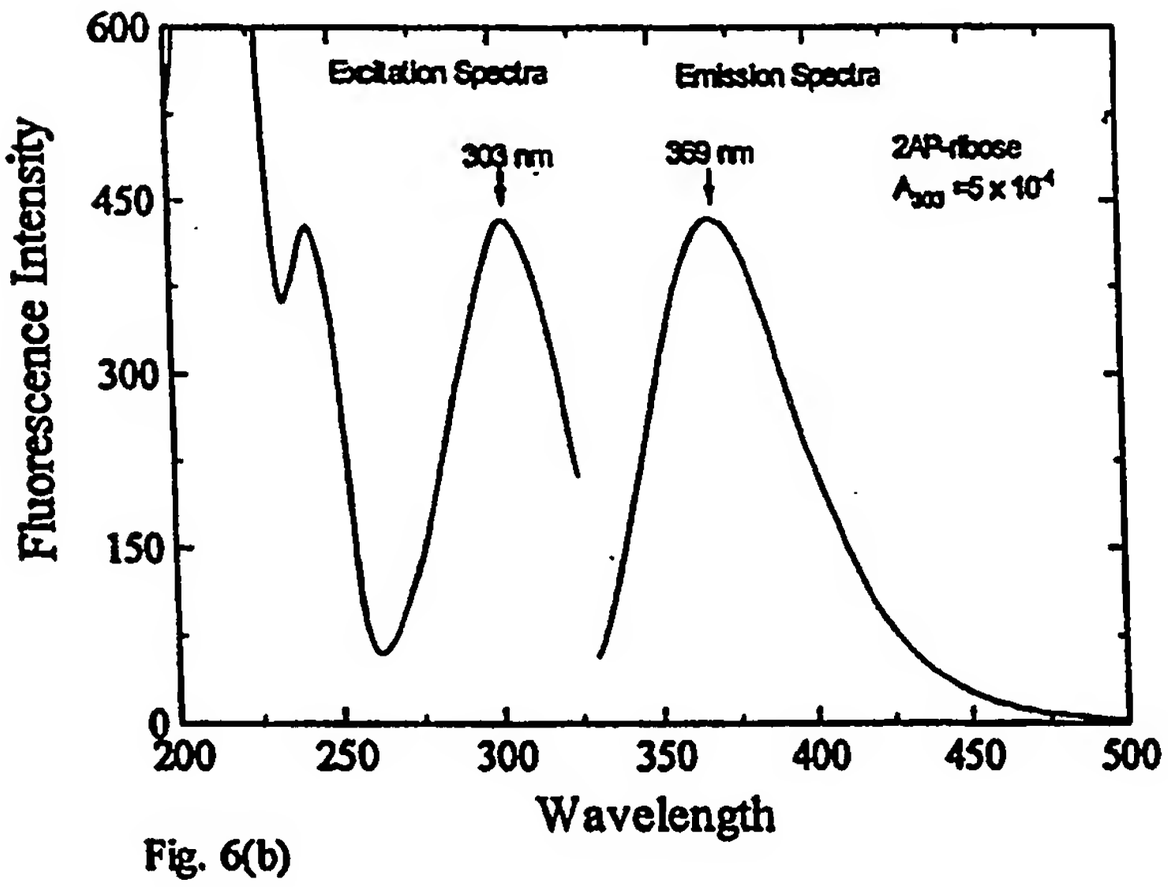

600
Excitation Spectra
Emission Spectra
303 nm
369 nm
2AP-ribose
$A_{303} = 5 \times 10^{-4}$
Fluorescence Intensity
450
300
150
0
200
250
300
350
400
450
500
Wavelength

Fig. 6(b)

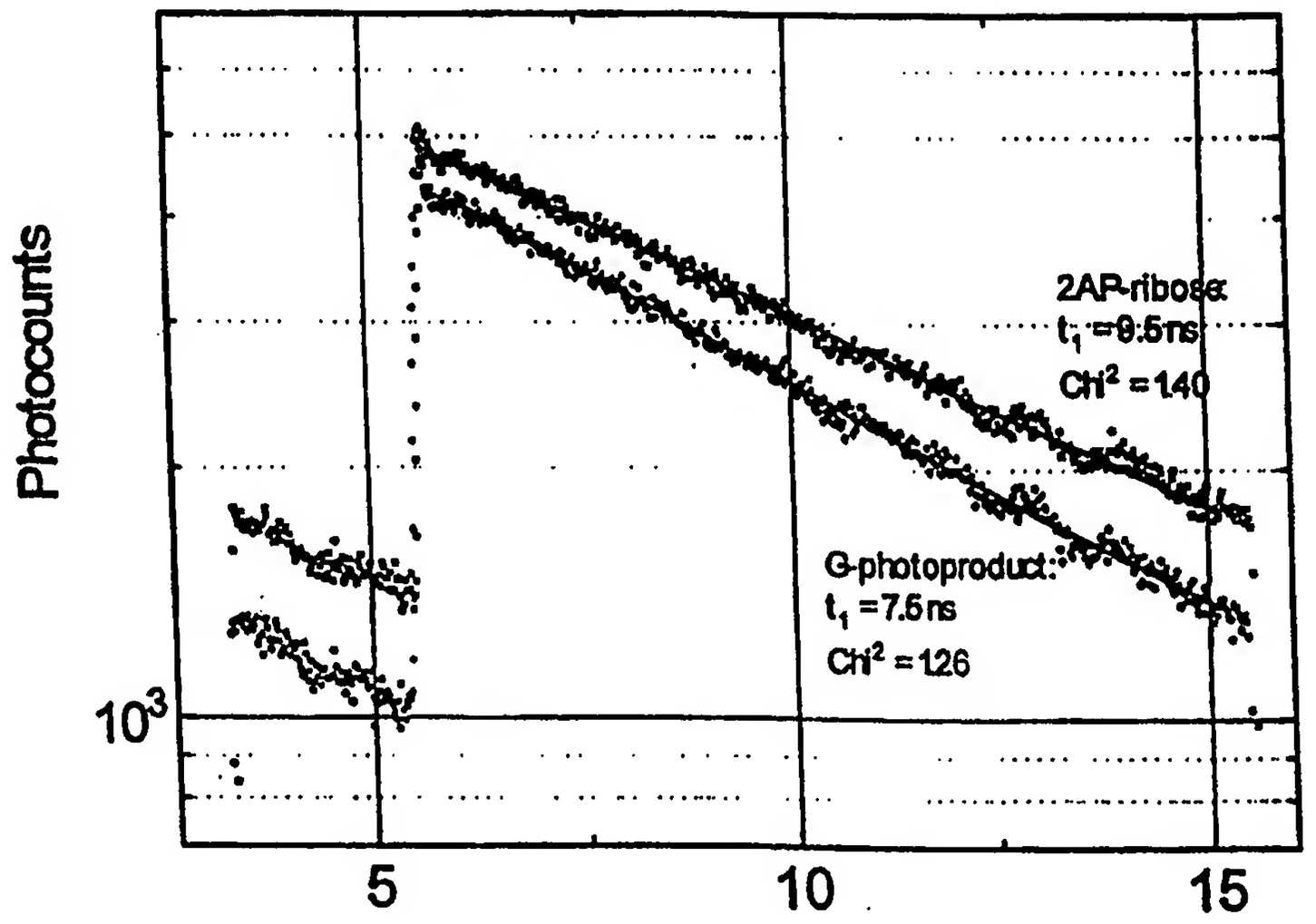

Photocounts
10^3
2AP-riboside
t_1 =9.5ns
Chi^2 =140
G-photoproduct
t_1 =7.5ns
Chi^2 =126
5
10
15
Time (ns)

Fig. 7

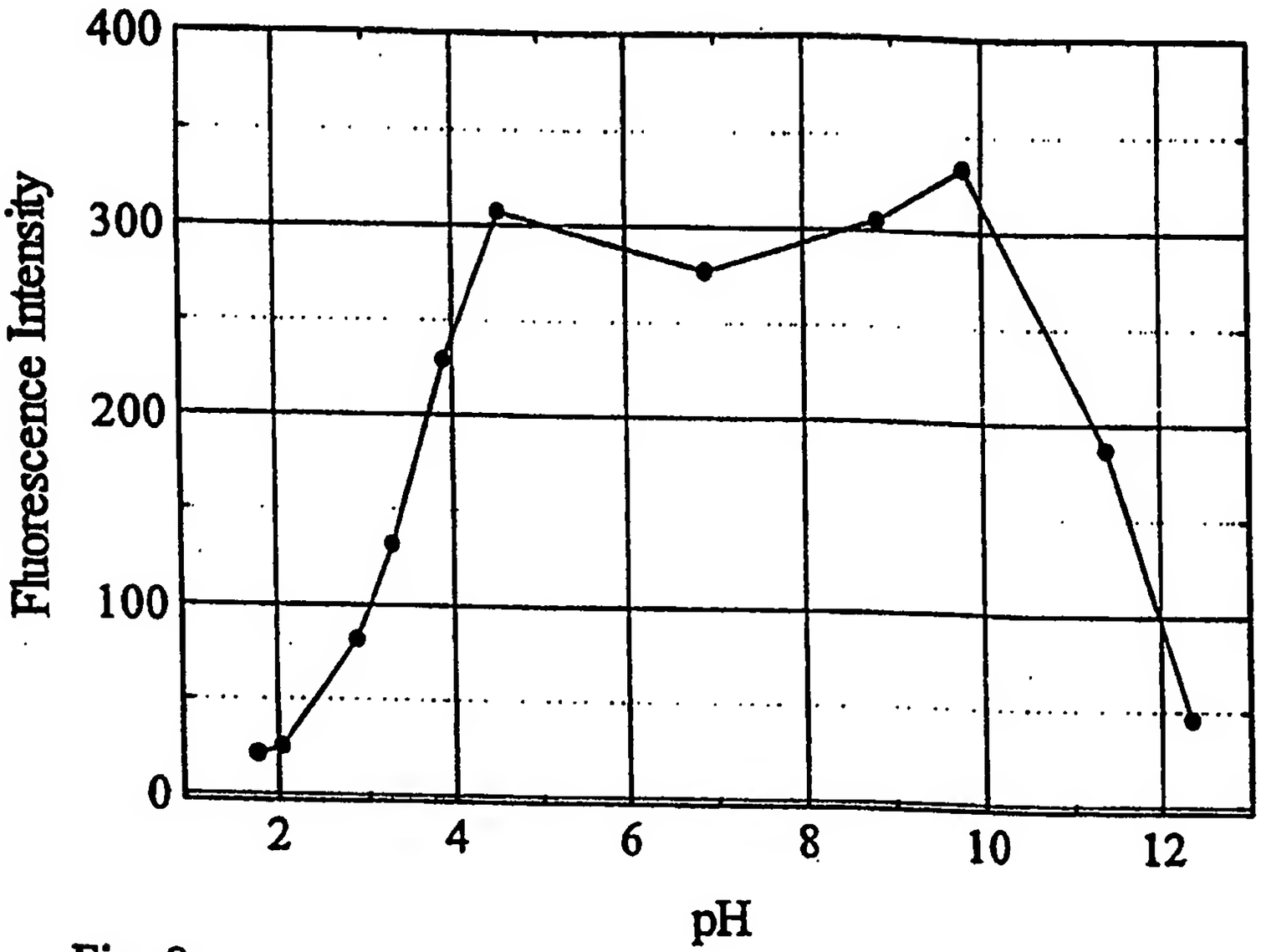
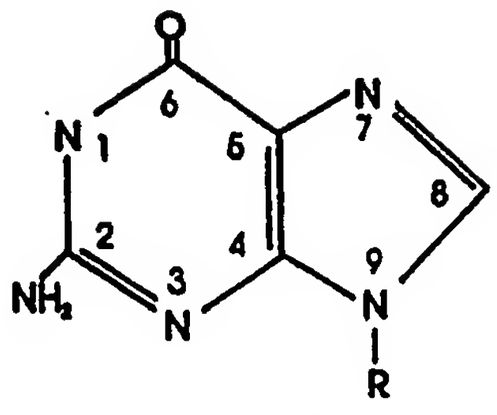
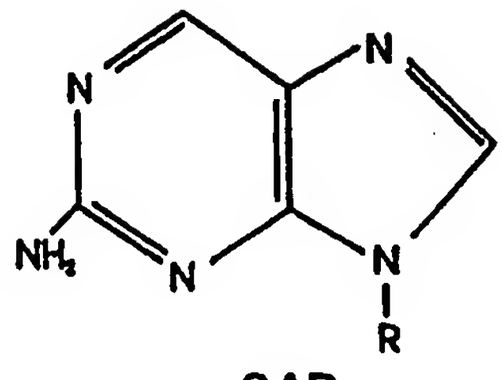


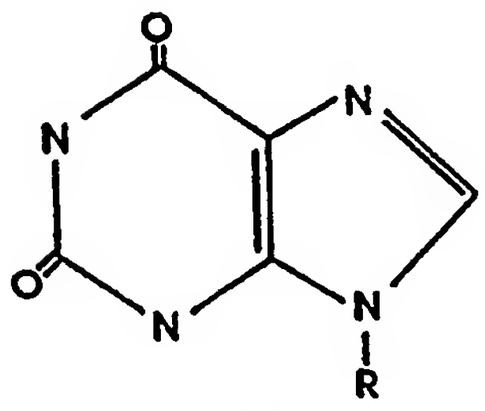
Fig. 8



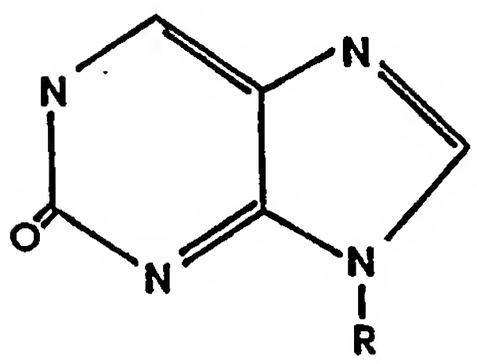
dGMP



2AP



XMP



Iso-inosine

FIG. 9

Fig. 10(a)

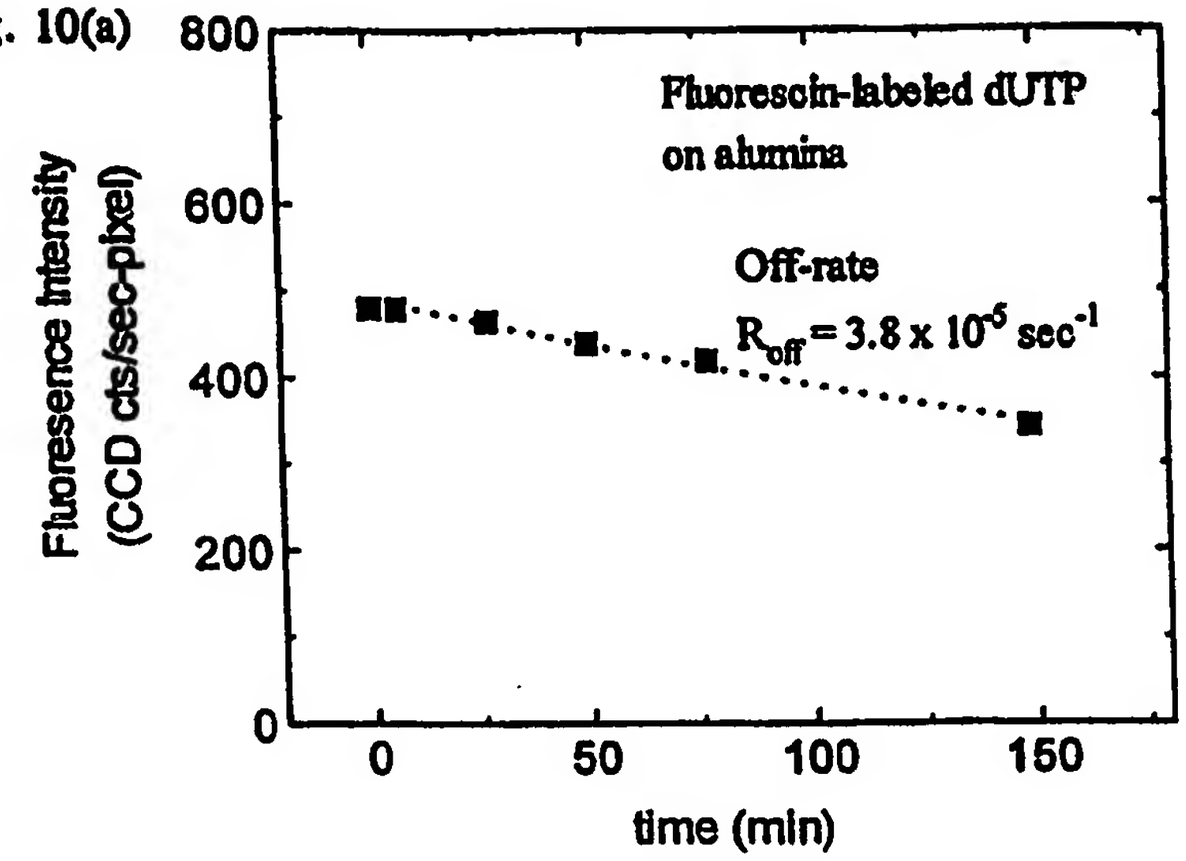
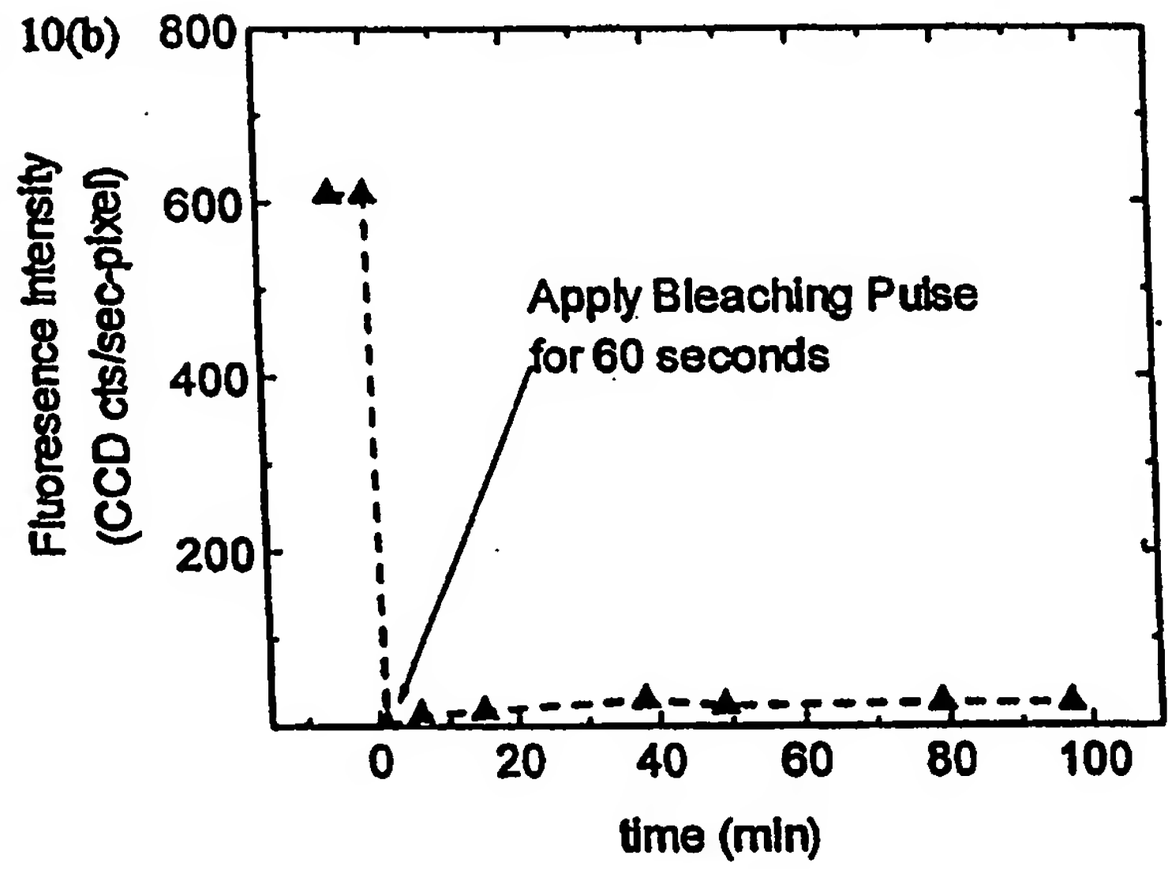


Fig. 10(b)



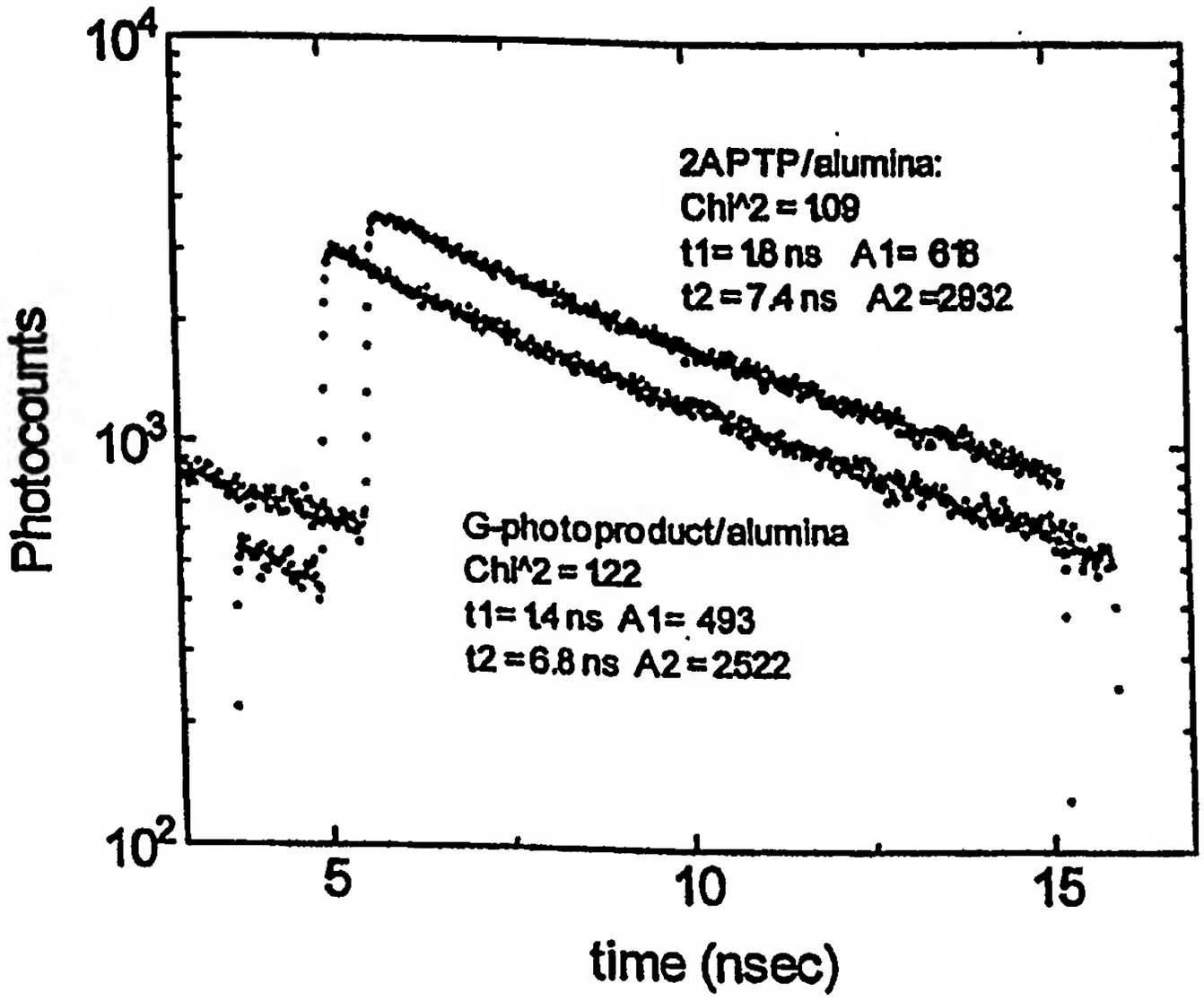

2APTP/alumina:
Chi^2 = 109
t1= 18 ns A1= 618
t2 = 7.4 ns A2 =2932
G-photoproduct/alumina
Chi^2 = 122
t1= 14 ns A1= 493
t2 = 6.8 ns A2 = 2522
Photocounts
10^4
10^3
10^2
5
10
15
time (nsec)

FIG. 11

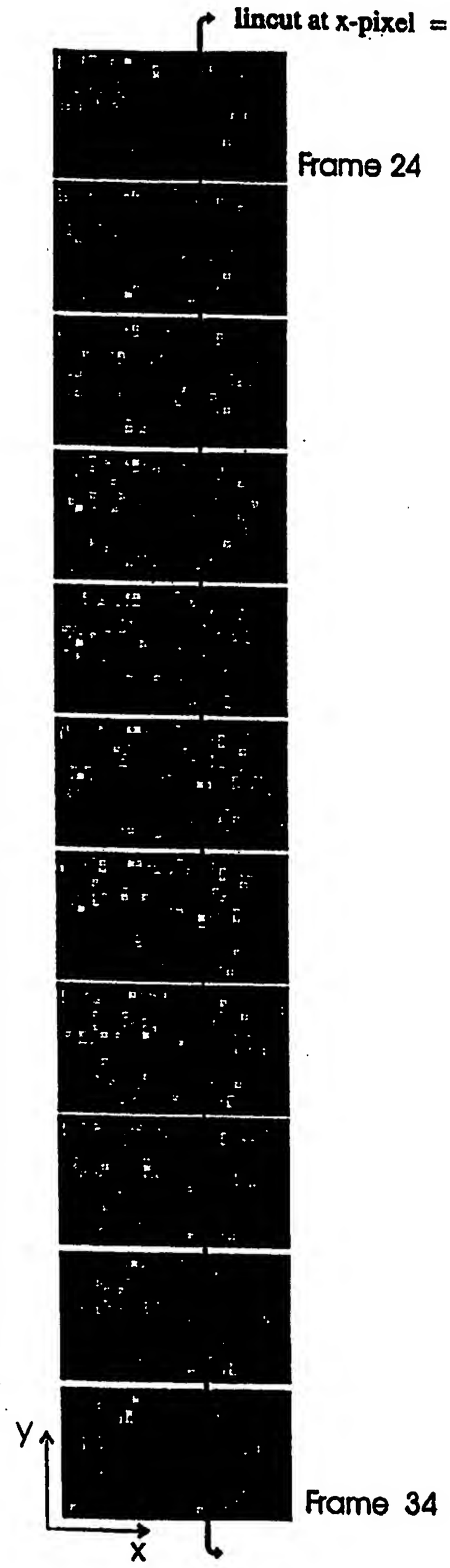


FIG. 12(a)

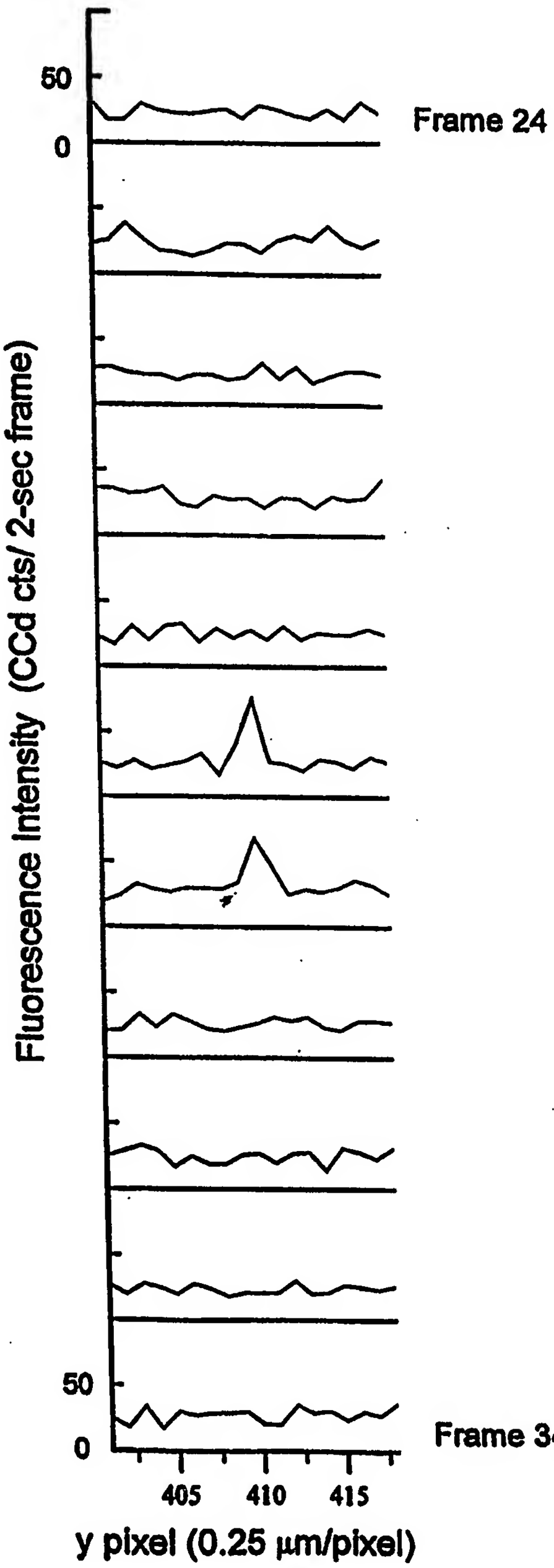

50
0
Frame 24
Fluorescence Intensity (CCd cts/ 2-sec frame)
50
0
Frame 34
405
410
415
y pixel (0.25 µm/pixel)

FIG. 12(b)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/18817

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12Q 1/68; C12P 19/34; C07H 21/00, 21/02
US CL :435/6, 91.1, 91.2; 536/22.1, 25.3

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 91.1, 91.2; 536/22.1, 25.3

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, STN
search terms: nucleotides, sequencing, photoproducts, radiation, electromagnetic, light

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 5,187,085 A (LEE et al.) 16 February 1993, see entire document.	1-15
Y	US 4,729,947 A (MIDDENDORF et al.) 08 March 1988, see entire document.	1-15
Y	US 5,332,666 A (PROBER et al.) 26 July 1994, see entire document.	1-15

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 06 NOVEMBER 1998	Date of mailing of the international search report 21 DEC 1998
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JEZIA RILEY Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★